

Analytical Profiles of Drug Substances

Volume 8

Edited by

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PREFACE

Although the official compendia list tests and limits for drug substances related to identity, purity, and strength, they normally do not provide other physical or chemical data, nor do they list methods of synthesis or pathways of physical or biological degradation and metabolism. For drug substances important enough to be accorded monographs in the official compendia, such supplemental information should also be made readily available. To this end the Pharmaceutical Analysis and Control Section, Academy of Pharmaceutical Sciences, has undertaken a cooperative venture to compile and publish *Analytical Profiles of Drug Substances* in a series of volumes of which this is the seventh.

The concept of analytical profiles is taking hold not only for compendial drugs but, increasingly, in the industrial research laboratories. Analytical profiles are being prepared and periodically updated to provide physicochemical and analytical information of new drug substances during the consecutive stages of research and development. Hopefully, then, in the not too distant future, the publication of an analytical profile will require a minimum of effort whenever a new drug substance is selected for compendial status.

The cooperative spirit of our contributors has made this venture possible. All those who have found the profiles useful are requested to contribute a monograph of their own. The editors stand ready to receive such contributions.

Thanks to the dedicated efforts of Dr. Morton E. Goldberg, a long cherished dream has come to fruition with the publication of *Pharmacological and Biochemical Properties of Drug Substances*, M. E. Goldberg, editor, published by APhA Academy of Pharmaceutical Sciences. This new series supplements the comprehensive description of the physical, chemical, and analytical characteristics of drug substances covered in *Analytical Profiles of Drug Substances* with the equally important description of pharmacological and biochemical properties.

Drug substances appearing in the new series will be cross-referenced in the cumulative index.

The goal to cover all drug substances with comprehensive monographs is still a distant one. It is up to our perseverance to make it a reality.

Klaus Florey

ASPIRIN

Klaus Florey

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1. Introduction

1.1 Foreword

The writing of an analytical profile of aspirin, this drug of drugs, poses two major dilemmas. The first is in the name itself. There are many countries where Aspirin is still a trade-name of the German firm Bayer AG, and Acetylsalicylic Acid is used as the generic. Yet, I decided to use the former because it is the U.S.P. and B.P., the better known worldwide and the more elegant name.

Aspirin has now been available for close to 80 years, and its usefulness and popularity are undiminished. Consequently, the literature is voluminous and also undiminished. A complete coverage would be beyond the scope of an analytical profile. I have endeavored to cover the newer literature as comprehensively as possible and have included only those older references which I found of historical interest. To all those who have labored in the vineyard of aspirin and who go unreferenced in this profile, I tender my sincere apologies.

1.2 History

The documented facts of the discovery of aspirin are quickly told. It was synthesized by the German chemist Felix Hoffmann (1868-1946) in the laboratories of Farbenfabriken Bayer, Elberfeld, Germany in 1897 (Fig. 1). The compound was tested pharmacologically by H. Dreser¹ and clinically among others by Wohlgenuth² and Witthauer³ who documented the antirheumatic, antipyretic and analgesic properties free of the undesirable side effects of salicylic acid.

Apparently, there was some initial reluctance at Bayer to market the new compound since it was thought that the field was already crowded with new drugs. But opposition faded when the new drug got the support of Carl Duisberg, then the general manager of Bayer. Duisberg, of course, was the great chemist and industrialist who built Bayer into the chemical giant of world renown. After the inspired trade name Aspirin, a contraction of acetyl and "spirsäure" (salicylic acid), was coined in the offices of Bayer - Euspirin was also considered and fortunately discarded⁴ - it was marketed in tablet form in 1899 and conquered the world.

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Dr. Hoffmann

Acetylsalicylsäure.

Wiegen 10g, Salicylsäure mit 15g, Acetanhydrid 3
 Stunden unter Rückfluß, so ist die S. ganzlich
 acetyliert. Auf demselben ist Essigsäure abdestillieren lassen
 in Wasser, die aus 660 kochender 136° kochen
 (Literaturangabe ist 138°). Der Jägerhof für die
 in Literatur gibt das reine Acetylsäure. Keine
 essigsaure Salze mehr, weil sie sich leicht durch
 Säure entzersetzt. Auf die physikalischen Eigenschaften
 von reinem Acetylsäure ist die Bestimmung unterworfen
 ist die Acetylsäure entzersetzt von der Säure
 und auf die entzetzten Säure auf die Säure

CD

Eiberfeld, den 10. Mai 1897

Hoffmann

Figure 1. Laboratory notebook entry of Felix Hoffmann, describing his first preparation of aspirin. The initials CD on the page are those of Carl Duisberg. (Courtesy of Bayer A.G., Leverkusen, Germany)

What motivated Hoffmann to undertake this momentous synthesis? Legend has it that he wanted to help his father who was suffering from rheumatism and who was no longer able to tolerate sodium salicylate, then widely used in rheumatic and arthritic diseases. Salicylic acid occurs naturally in several plants. The analgesic and antipyretic properties of willow bark were already known in antiquity to Hippocrates and the blossoms of *spiraea ulmaria* (meadow sweet) were used in the middle ages. Salicylic acid was crystallized from willow bark extracts in the early years of the last century, and Kolbe, in 1859, was able to synthesize it from sodium phenolate and carbon dioxide. His student von Heyden worked out a commercially feasible process and started a factory to produce salicylic acid which made possible its widespread use in rheumatic diseases. However, its bad taste, stomach irritation and other side effects were a strong incentive to search for derivatives which retained its efficacy without its disadvantages. Acetylation of the hydroxyl group was one of the logical modifications. Acetylated salicylic acid had already been described three times in the literature (see Section 3). Von Heyden and possibly also Merck, Darmstadt, are reputed to have experimented with aspirin without being able to produce the pure drug.

At the time when Felix Hoffmann prepared pure aspirin successfully in the Bayer laboratories, one of his colleagues was Arthur Eichengrün, who had been hired by Carl Duisberg in 1896, while Hoffmann had been hired in 1894. Eichengrün, as an old man, had to undergo the horrors of the infamous Nazi concentration camp in Theresienstadt which he survived. In 1949, Eichengrün published his memoirs relating to the invention of aspirin⁵ which was then a half-century old. He claimed that it was he who told Hoffmann to prepare acetylsalicylic acid. Acetylation certainly was on Eichengrün's mind, since he had also experimented successfully with the acetylation of cellulose about the same time. He went on to fame as the inventor and developer of rayon and safety film. Eichengrün also claimed that another colleague of Bayer, the pharmacologist Dreser, opposed clinical trials. However, the memory of the 82 year-old Eichengrün must have been faulty when he

wrote these rather bitter reminiscences concerning Hoffmann's, Dreser's and his own role in the discovery of aspirin because, in 1913, Eichengrün wrote a chapter on "The Pharmaceutical Research Laboratory" in the book History and Development of Farbenfabriken Bayer, Vorm. Friedr. Bayer & Co., Elberfeld by F. Fischer, 1913⁴, where he laid no paternity claim to aspirin and described Dreser's role correctly. The pertinent passage (p. 412) translates as follows: "Acetylsalicylic acid, prepared by Felix Hoffmann rested unnoticed for 1½ years among the preparations rejected by the pharmacological laboratory until in 1898, during unrelated investigations, Dreser's attention was again drawn to it. On account of the observation that the acetyl compound was increasing cardiac activity in contrast to salicylic acid itself, he recommended a clinical trial of the product...."

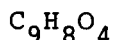
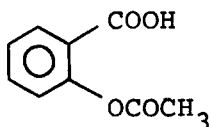
Felix Hoffmann did not publish his version of the discovery, nor did he obtain a German patent, since the synthesis had been previously described. Farbenfabriken Bayer did obtain a U.S. Patent⁶ in 1900 which named him as the inventor. Chemical Abstracts reveal no subsequent publications by him, nor is there any record that he was publicly honored for his contribution. However, in 1899, he was appointed director of the pharmaceutical research and marketing division of Bayer. He retired in 1928⁷.

In many ways, the story of the discovery of aspirin is typical for the way in which new drugs are invented and developed in pharmaceutical research laboratories, where many individuals have to make a contribution and where it is often difficult to fathom completely what thought processes, suggestions and interactions lead to a successful new drug.

2. Description

2.1 Name, Formula, Molecular Weight

Aspirin is acetylsalicylic acid, also salicylic acid acetate and 2-(acetyloxy)-benzoic acid (50-78-2). The last name is currently popular in Chemical Abstracts.



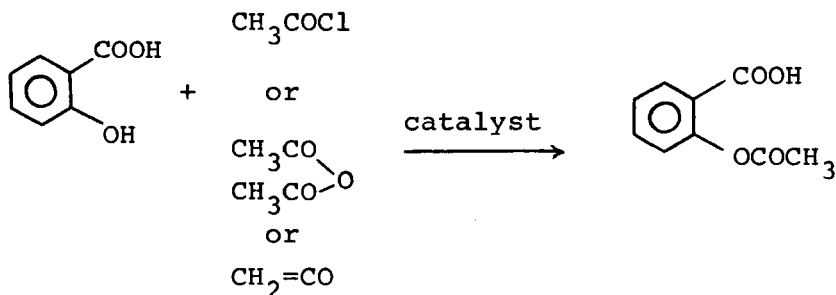
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2.2 Appearance, Color, Odor

Aspirin is a white, crystalline powder. It is odorless but might have a faint odor of acetic acid.

3. Synthesis

The first synthesis of aspirin is credited to Gerhardt⁸ in 1853. Gerhardt was investigating mixed organic acid anhydrides and, among others, reacted acetylchloride with sodium salicylate. He obtained a solid product, undoubtedly impure acetylsalicylic acid, which immediately and without further characterization he hydrolyzed with aqueous sodium carbonate to salicylic and acetic acids. Next it was prepared by reaction of salicylic acid with acetylchloride by H. von Gilm⁹ in 1859, who described a crystalline product. In 1869, K. Kraut¹⁰ had a student, A. Prinzhorn, prepare acetylsalicylic acid by the methods of Gerhardt and von Gilm and obtained an identical product by both methods with a reported melting point of 118.5°. Kraut also correctly observed that the product is not an acid anhydride as assumed by Gerhardt but rather a phenolic ester. Felix Hoffmann⁶ used acetic anhydride for its preparation.



Essentially, all methods of synthesis are variations of the reaction of acetylchloride, acetic anhydride or ketene¹¹ with salicylic acid using a variety of catalysts such as pyridine¹² or sulfuric acid¹³ and reaction conditions (c.f. 14). The preparation of aspirin labeled with a ¹⁴C-labeled acetyl group has also been reported.¹⁵ Efforts to improve the commercial processes continue to the present day.

4. Physical Properties

4.1 Spectra

4.11 Infrared

The assignment of the KBr infrared spectrum (Figure 2) of aspirin (U.S.P. reference standard #0675-F-4) is summarized in Table 1.¹⁶ It agrees essentially with a spectrum published previously.¹⁷ A reflection spectrum has also been presented.¹⁸

TABLE 1

Infrared Spectrum Interpretation

<u>Wavelength (cm⁻¹)</u>	<u>Assignment</u>
2300-2500	carboxyl OH
1760	vinyl ester C=O
1690	aromatic acid C=O
1610 } 1580 } 1490 }	aromatic C=C stretch
1220 }	
1190 }	
760	=C-O (acid and ester)
	ortho subst. phenyl C-H bending

4.12 Ultraviolet

Aspirin in 0.1N sulfuric acid¹⁹ and in dilute trichloroacetic acid²⁰ exhibits maxima at 229 nm (E_1 484) and 276 nm (E_1 65.5).¹ In chloroform a maximum was found at 277 nm (E_1 68).²¹

4.13 Fluorescence - Phosphorescence

The native fluorescence of aspirin, in contrast to salicylic acid, is a weak one and has been studied only recently.²² Excitation wavelength maximum is at 280 nm and emission maximum is at 335 nm. Maxima for salicylic acid are at 308 and 450 nm respectively.

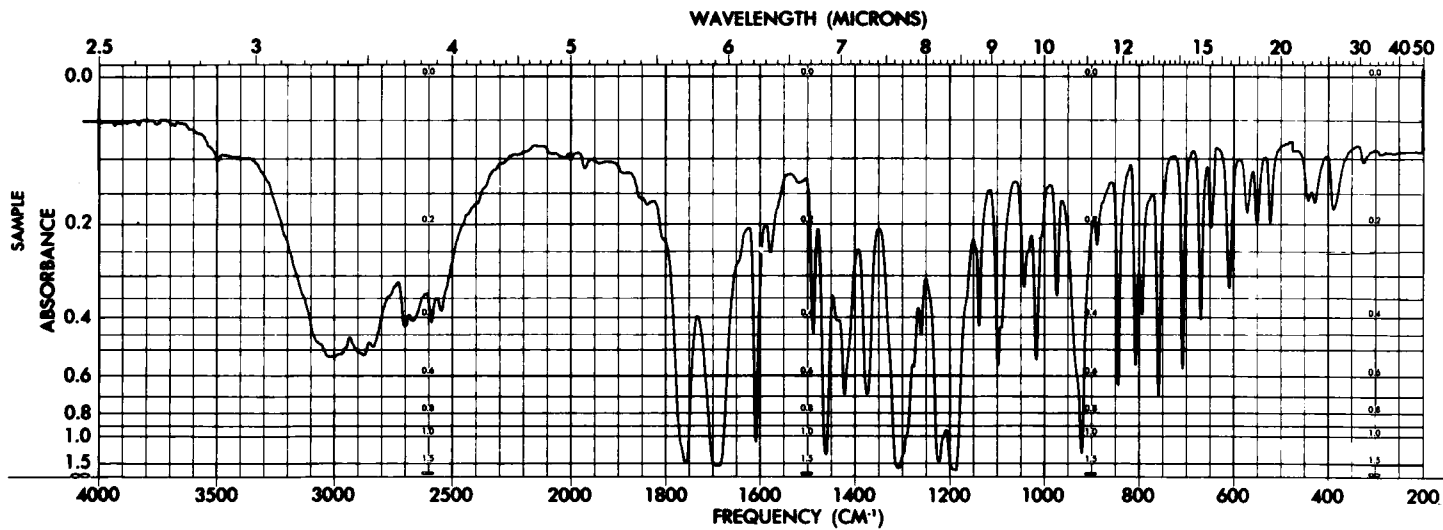


Figure 2. Infrared Spectrum of Aspirin (U.S.P. Reference Standard)
KBr pellet. Instrument: Perkin-Elmer Model 621

The phosphorescence emission maximum was found at 410 nm.²³

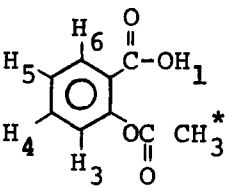
4.14 Raman

Raman spectra are described and discussed in the following references: 24, 25

4.15 Nuclear Magnetic Resonance

The 60 and 100 MHz proton magnetic resonance spectra of aspirin have been published as part of analytical²⁶⁻²⁹ and biochemical studies.^{30,31}

The 100 MHz pmr spectrum of a deuteriochloroform solution containing tetramethylsilane as an internal reference was obtained on a Varian Associates XL-100-15 spectrometer equipped with a Nicolet pulsed Fourier accessory.³² (Figure 3.) The rms error for the experimental and calculated spectra shown in Figure 4 is 0.2. The proton assignment is shown below.

	δ	
	H ₁ 12.04	J _{3,4} = 8.05Hz
	H* 2.34 (3H)	J _{3,5} = 1.34Hz
	H ₃ 7.13	J _{3,6} = 0.3Hz
	H ₄ 7.61	J _{4,5} = 7.80Hz
	H ₅ 7.33	J _{4,6} = 1.74Hz
	H ₆ 8.11	J _{5,6} = 7.96Hz

The differences in the pmr spectrum previously reported in aqueous media³⁰ are attributed to the solvent used. The proton-proton couplings of the aryl protons are virtually identical, however.

The fully decoupled ¹³C-NMR spectrum of aspirin in CD₃OD (200 mg/ml) is shown in Figure 5. The spectrum was obtained on a Varian XL-100-15 NMR spectrometer equipped with a Transform Technology TT-100 FT data system. The data represent the transformation of a 400 pulse FID obtained using 4096 data points with a 15 second delay time between accumulations.³³ Peaks a-i (Figure 5) arise from the nine carbons of aspirin. The seven peak multiplet centered at $\delta=49.0$ ppm

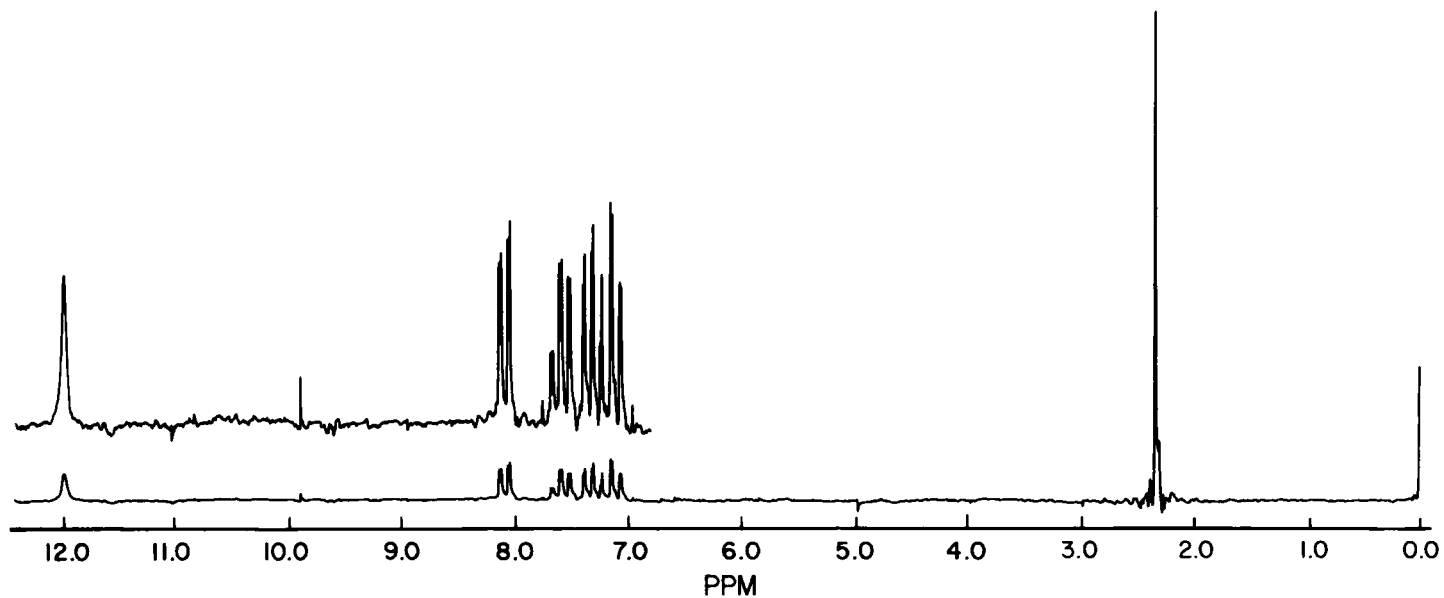


Figure 3. 100 MHz PMR Spectrum of Aspirin. Instrument: Varian XL-100-15

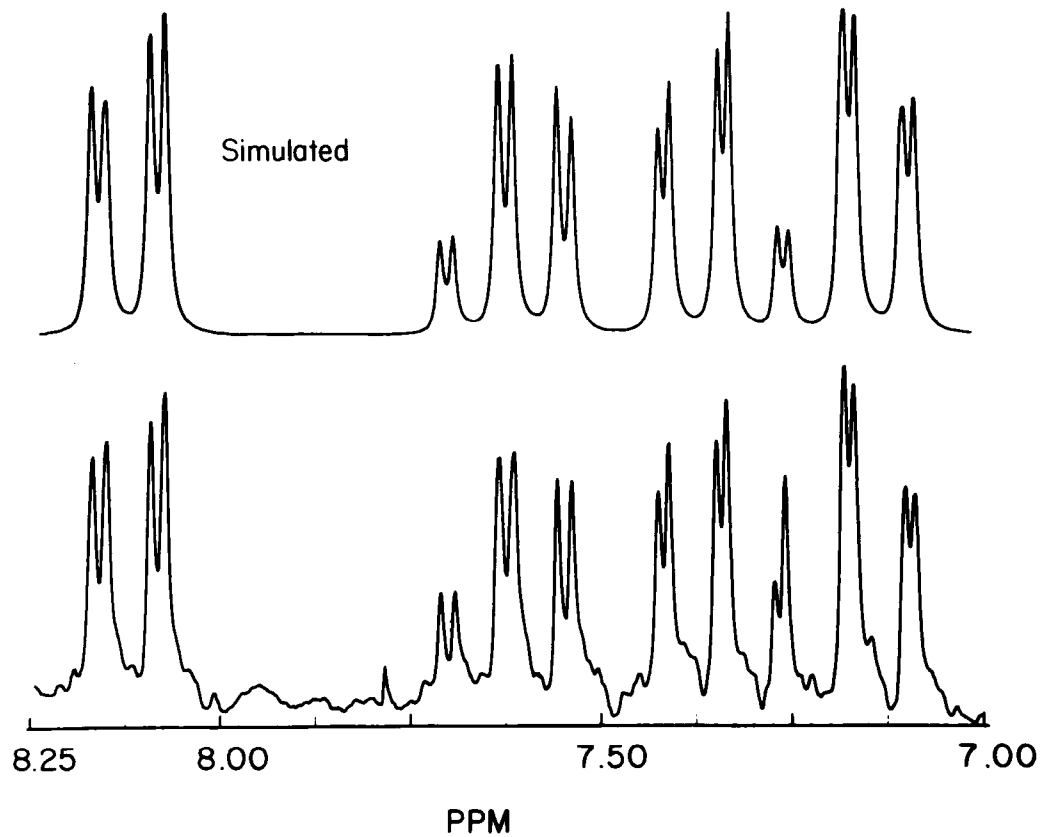


Figure 4. Simulated and calculated PMR Spectra of Aspirin.

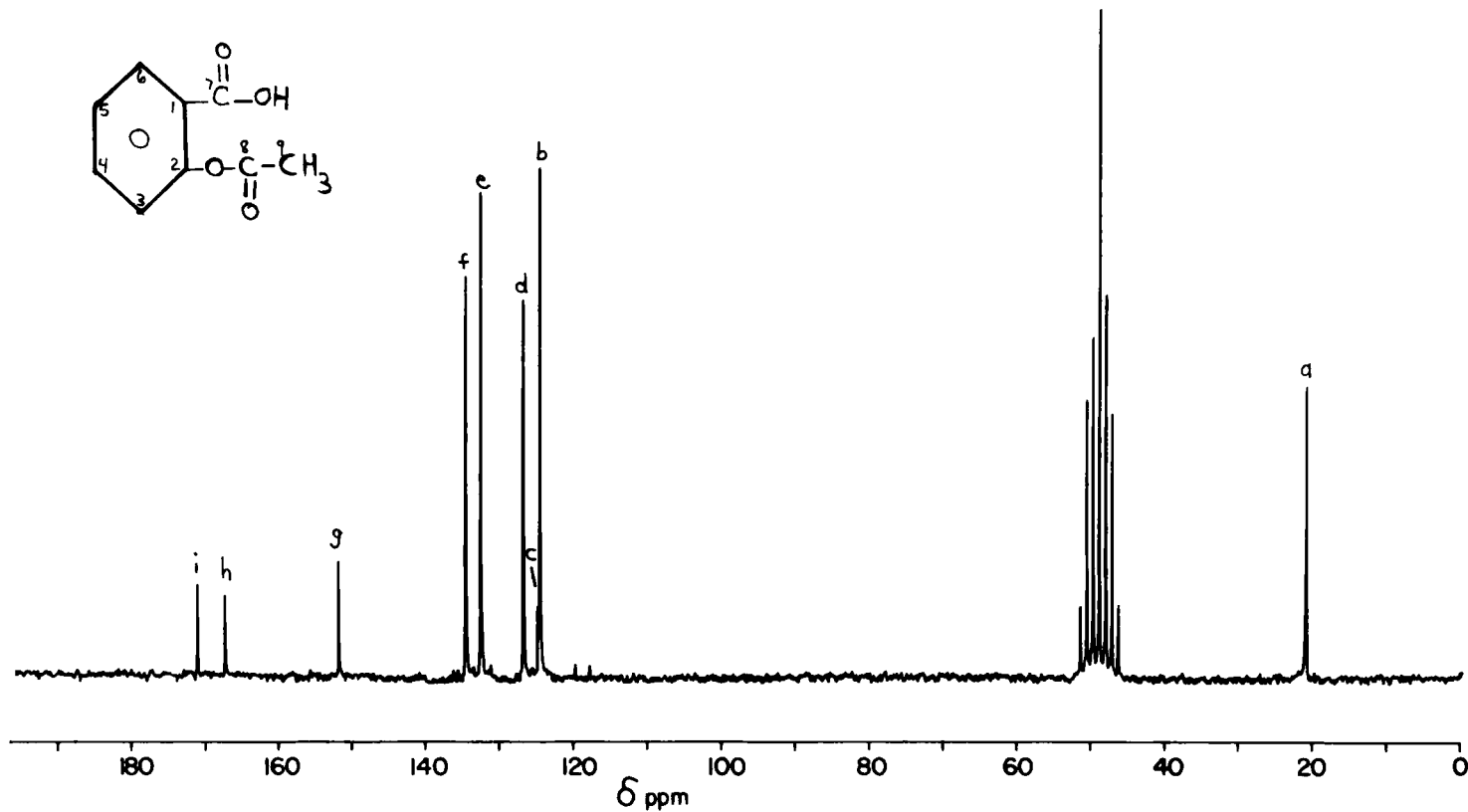


Figure 5. ^{13}C -NMR Spectrum of Aspirin in CD_3OD . Instrument: Varian XL-100-15

arises from the solvent. Assignment of each of the peaks can be made on the basis of their chemical shifts and the coupling information summarized in Table 2. Peak a is the sole peak in the aliphatic region of the spectrum and is, therefore, assigned to methyl carbon (C-9). As expected, the fully coupled spectrum exhibits four peaks in this region with a $^1J_{CH}$ of 130 ± 2 hz. Peaks b, d, e and f can be assigned to the four singly protonated aromatic carbons. Each is strongly coupled to one proton with a $^1J_{CH}$ of 165 ± 2 hz. Weak long range coupling can also be observed. Peak b, d, e and f can be assigned to carbons 3, 5, 6 and 4, respectively by comparison of their chemical shifts to values predicted on the basis of substituent effects observed in model systems. Peaks c, g, h and i must arise from non-protonated carbons since they do not exhibit appreciable splitting in the fully coupled spectrum. Chemical shift predictions based on substituent effects enable assignment of peaks c and g to the C-1 and C-2 aromatic ring carbons, respectively. The remaining peaks h and i must arise from the carbonyl carbons. Comparison of their respective chemical shifts to those of model compounds suggest their assignment to the carboxyl (C-7) and acetoxy carbonyls (C-8), respectively. This assignment is confirmed by the observation of a long range coupling of peak i to the three protons of the methyl group.

TABLE 2
 ^{13}C -NMR Data for Aspirin

Peak	δ (ppm) ^a	Assignment Carbon #	$^1J_{CH}$ ^b	Multiplicity ^b
a	21.6	9	130	(4)
b	124.6	3	164	(2)
c	124.9	1	c	m
d	126.9	5	165	(2)
e	132.6	6	165	(2)
f	134.7	4	163	(2)
g	151.9	2	c	m
h	167.4	7	c	m
i	171.2	8	c	m (4)

a) ppm from TMS external via the relationship
 $\delta(\text{CD}_3\text{OD}) = 49.0$ ppm.

b) obtained from the fully coupled spectrum.

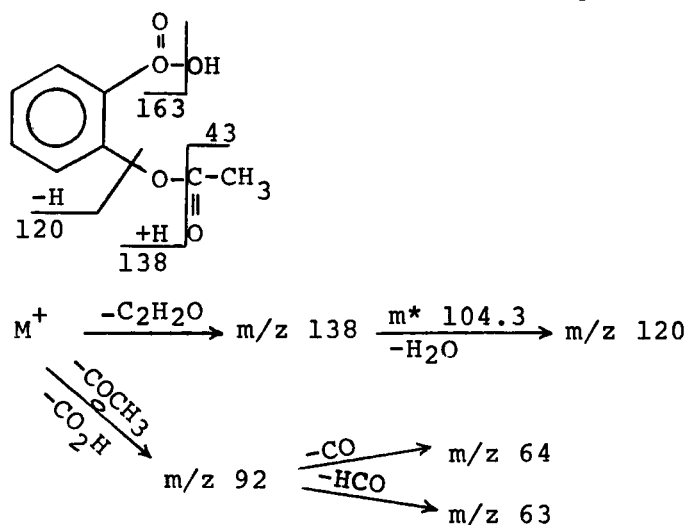
- c) none observed.
- m) weakly coupled multiplets due to long range effects.

4.16 Mass

The low resolution mass spectrum was obtained on an AEI MS-902 double-focussing mass spectrometer equipped with a frequency-modulated analog tape recorder at a source temperature of 100° C. above ambient (approximately 130° C.).³² By adjusting the sample flow and the electron multiplier gain, the maximum sensitivity was obtained without clipping the most intense peak. The recorded analog spectrum was processed on a PDP-11.³⁴ Except for differences in the intensity of the molecular ion (M^+), the intensities shown in Figure 6 are virtually identical to the previously published spectrum.³⁵ Differences could be ascribed to instrument design, source temperatures or even source design.

The assignment of a number of fragment ions of the mass spectrum is shown in Figure 7.

Figure 7.



The metastable ion at $m/z\ 104.3$ supports the loss of the elements of water from the $m/z\ 138$ rearrangement ion.

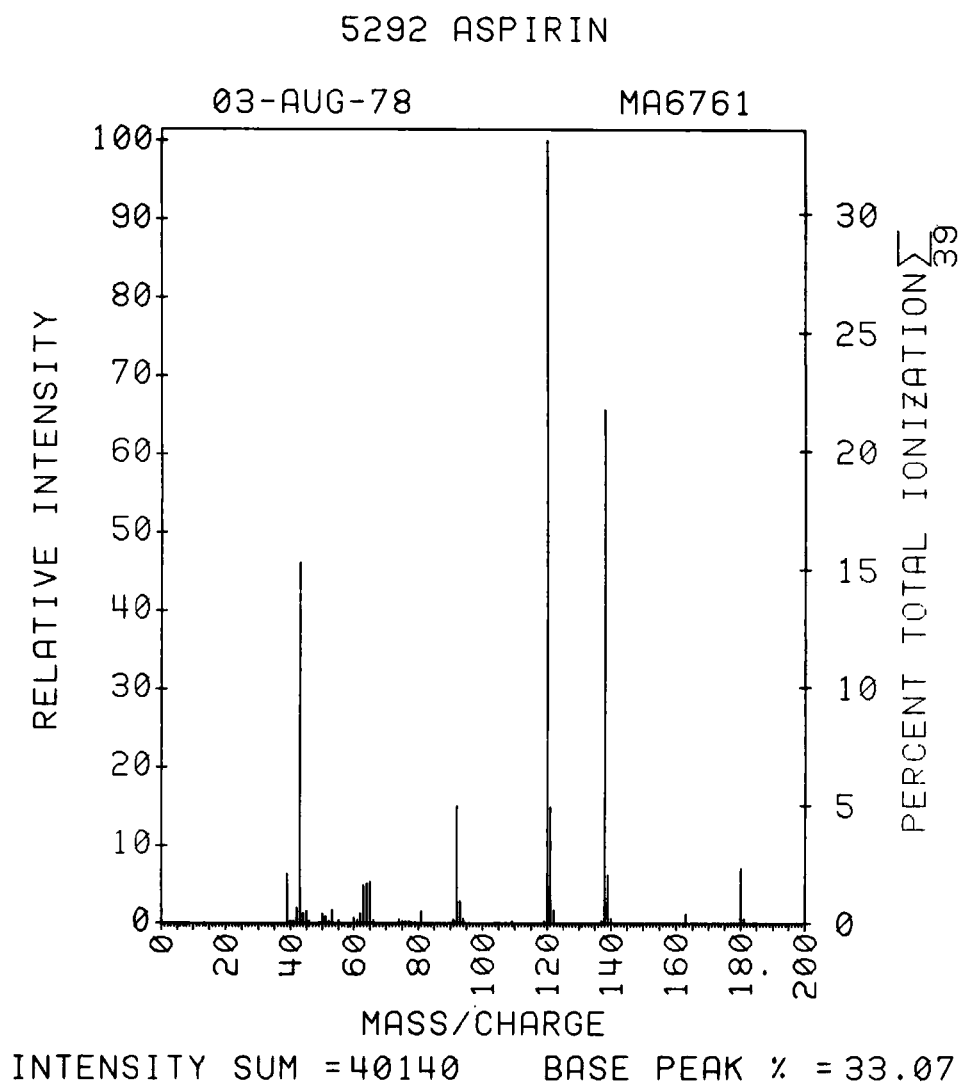


Figure 6. Mass Spectrum of Aspirin.
Instrument: AEI MS 902

The mass spectrum of aspirin has been used as an aid in the rapid identification of toxic materials isolated from urine, blood or gastric aspirates of drug abuse patients.^{36,37}

4.2 Solid Properties

4.21 Melting Range

The melting point of aspirin is nothing very definitive having variously been given between 118-144³⁸ and a good deal of work has been done to get the best methods for compendial use.³⁹⁻⁴³ Early commercial preparation melted around 135⁴⁴. The European Pharmacopeia⁴⁵ gives a melting point of 141^o to 144^o as determined by the instantaneous method. For further discussion, see Polymorphism (Section 4.242).

4.22 Differential Thermal Analysis

When aspirin (USP reference standard) was heated at a rate of 15^o/min. in air, a single endotherm was observed with a T onset = 134^o and T peak = 139^o.⁴⁶ DTA and TGA patterns of aspirin have also been previously studied⁴⁷ and used for forensic drug identification.⁴⁸

4.23 Thermogravimetric Analysis

When aspirin (USP reference standard) was heated at 20^o/min. and a N₂ flow of 20 cc/min., no weight loss was observed at less than 130^o.⁴⁶

4.24 Crystal Properties

4.241 Single Crystal X-Ray Diffraction

The crystal structure of aspirin was determined by Wheatley.⁴⁹ The monoclinic crystals have a space group of P₂₁/C. The dimension of the unit cell are: a=11.446Å; b=6.596Å; c=11.388Å; β=95^o 33'; n=4.

4.242 Powder X-Ray Diffraction

The powder x-ray diffraction pattern of aspirin is presented in Table 3 and Figure 8.⁵⁰

4.243 Polymorphism

In 1968, Tawashi⁵¹ claimed that aspirin exists in several polymorphic forms.

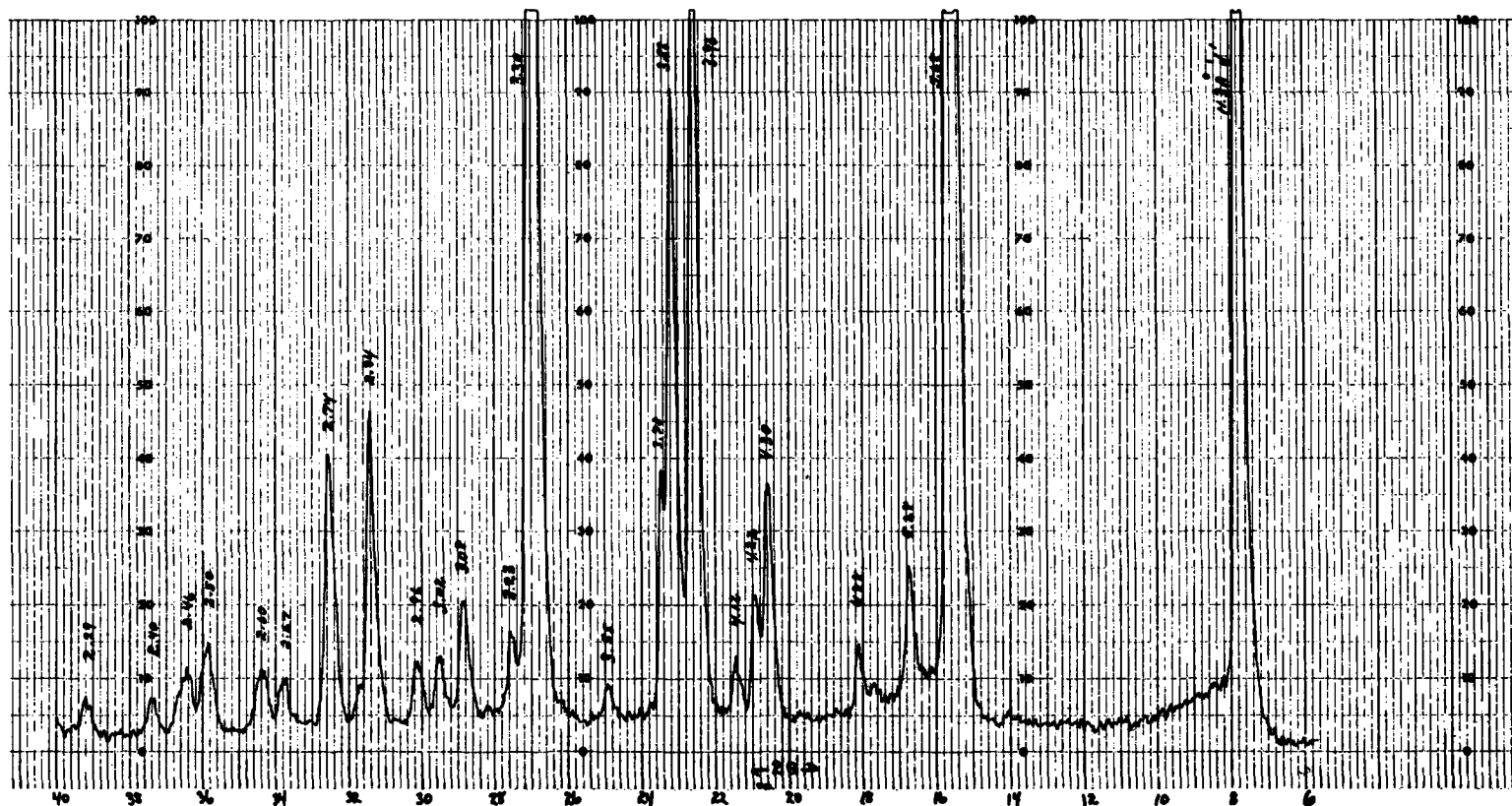


Figure 8. Powder X-Ray Diffraction Pattern of Aspirin (U.S.P. Reference Standard). Instrument: Norelco

TABLE 3Powder X-Ray Diffraction Pattern of Aspirin
(U.S.P. Reference Standard)

<u>20</u> <u>(Deg.)</u>	<u>D (Å)</u>	<u>Relative</u> <u>Intensity</u>	<u>20</u> <u>(Deg.)</u>	<u>D (Å)</u>	<u>Relative</u> <u>Intensity</u>
7.80	11.3	0.539	27.56	3.23	0.054
14.09	6.25	0.033	28.85	3.08	0.068
15.63	5.68	1.000	29.62	3.02	0.040
16.78	5.28	0.087	30.26	2.96	0.039
18.19	4.88	0.038	31.54	2.84	0.120
20.63	4.30	0.079	32.57	2.74	0.110
20.95	4.22	0.035	33.85	2.67	0.051
21.53	4.12	0.030	34.50	2.60	0.037
22.56	3.93	0.268	36.04	2.50	0.049
23.20	3.83	0.210	36.55	2.46	0.048
25.00	3.55	0.033	37.45	2.40	0.034
27.05	3.30	0.427	39.37	2.29	0.038

This started a flurry of activity. DeBisschop⁴³ claimed to have obtained three different crystal forms but stressed that the only stable one is the monoclinic one melting at 142° C.

However, the claim for true polymorphism of aspirin was questioned or refuted in several laboratories⁵²⁻⁵⁶ and can be best summarized in the words of G. Schwartzman:⁵⁷

"It is generally accepted that true polymorphism results in distinct optical and spectral properties. The data presented are entirely negative in these respects. The evidence accumulated agrees with the findings of Pfeiffer⁵⁵ and questions the formation of aspirin polymorphs. We believe that the different crystal habits were caused by the solvents used for crystallization. The dissimilar melting points are probably due to the poor transfer of heat caused by the larger crystal size or to possible crystal defects."

4.244 Optical Constants

Aspirin has been described as monoclinic, $a:b:c = 1.7322:1:1.7322$, $\beta=95^\circ 4.25'$. Indices for 576 $\mu\mu$ are: $d=1.5042$; $\beta=1.6424$

and $z=1.6554$; $2V=15^{\circ} 46'$. The optical plane is normal to (010) and lies in obtuse angle β .⁵⁸ Very similar constants are presented in reference 41.

4.245 Polarized Crystal Absorption Spectrum

The polarized absorption spectrum of a single crystal of aspirin was measured which indicated that aspirin may be spectroscopically treated as perturbed benzoic acid.⁵⁹

4.25 Calorimetry

The heat of combustion at constant volume was determined as 859.3 kcal/Mol.⁶⁰ Aspirin tablets make good samples for use in oxygen bomb calorimetry.⁶¹

4.3 Solution Properties

4.31 Solubility⁶²⁻⁶⁴

	g/ml
Water at 25°	0.0033
Water at 37°	0.01
Water at 100°	0.03
Ethanol	0.2 - 0.4
Chloroform	0.025 - 0.06
Carbon tetrachloride	0.0004
Ether	0.1 - 0.2
Abs. ether	sparingly soluble
Benzene	0.0033
Petroleum ether	insoluble

The solubility in polyethylene glycol 400 and in aqueous solution of other polyethylene glycols has been described.⁶⁵⁻⁶⁶ The effect of selected surfactants above and below the critical micelle concentration (CMC) on aspirin solubility⁶⁷ was studied.

4.32 Dissociation Constant (pKa)

In 1913, Springer and Jones⁶⁸ determined the dissociation in aqueous solution at various temperatures. At 25° they determined the dissociation constant as 2.8×10^{-4} (pKa 3.55). The Merck Index⁶² gives a value of 3.27×10^{-4} (pKa 3.49) at 25°.

When the apparent pKa was de-

terminated in DMF, using quaternary butyl ammonium hydroxide as the titrant, the pKa observed depended on the solvent of crystallization. From ethanol (m.p. 140-142°), a value of 8.99; from hexane (m.p. 121-124°), a value of 9.19 was obtained. The latter higher value was ascribed to internal hydrogen bonding of the carbonyl to the hydroxy group.⁶⁹ This apparent pKa should not be confused with the true pKa of 3.5 (see above).

4.33 Partition Coefficients

When aspirin was partitioned between buffers pH 1-7 and octyl alcohol, partition coefficients ranging from $k=17.7$ (pH 1) to $k=0.025$ (pH 7) were obtained.⁷⁰ Earlier, coefficients of 0.32 in toluene:water and 1.81 in chloroform:water were determined.⁷¹

4.34 Dielectric Constant, Dipole Moment

Dielectric constant:	Dipole Moment:	Ref.
~ 2.35	2.09	72
5 to 7	-	73
	5.65 calc.;	74
	4.36 observed	
	0.93 (acc. to	75
	Onsager by immersion)	

4.35 Radiation Absorption

The absorption coefficient of a collimated beam of ⁶⁰Co z-radiation was determined for aspirin. See reference 76 for details.

5. Methods of Analysis

5.1 Historical Synopsis

As the following pages of this section will show, there is hardly a new method of analysis which is not immediately tried for the determination of aspirin as such, or in formulations and biological fluids. The analysis of aspirin is intricately interwoven with that of salicylic acid, its precursor and degradation product. From the very first, residual salicylic acid was determined by the convenient reaction with ferric salts -- typical for phenols -- which give a violet complex with salicylic acid.

In spite of the plethora of methods, the

compendial approach to determine purity and strength of aspirin has been very conservative. A monograph for aspirin was introduced into U.S.P. not earlier than Volume X (1926) and has not been changed in its essentials for the last fifty years: Aside from such niceties as ash, carbonizable substances, chloride, sulfate and heavy metals, the mainstays then (U.S.P. X, 1926) and now (U.S.P. XIX, 1975) are: a) identification by a color test with ferric chloride after heating, saponification to salicylic acid, identified as a white precipitate and an odor test (ethyl acetate), b) residual salicylates as determined by a color matching test with ferric ammonium sulfate with a 0.1% limit and c) an assay involving saponification to salicylic and acetic acids and back titration of excess alkali with a purity specification of 99.5 to 100.5%.

The European Pharmacopeia⁴⁵ uses essentially the same analytical methods as U.S.P.

In contrast to aspirin itself, the U.S.P. monograph for aspirin tablets has undergone considerable changes. For some reason, U.S.P. does not use the ferric salt test for free salicylic acid, as does the British Pharmacopeia of 1973. Apparently, certain excipients such as citric and tartaric acid interfere with this reaction.⁷⁷ Already in 1913, a double titration method was developed⁷⁸ which was made an official method in 1926.⁷⁹ This method was used as the assay method when the aspirin tablets monograph was introduced into U.S.P. XII in 1942. For identification, the same two tests as for aspirin itself were prescribed then (U.S.P. XII) and now (U.S.P. XIX); however, due to the pioneering work of Higuchi, Banes, Smith and Levine in the '50's,⁸⁰ a test for non-aspirin salicylates was introduced using a siliceous earth column for separation from excipients and aspirin, and spectrophotometric finish at 306 nm. A limit of 0.3% is specified.

The same column method with different eluting solvents and a spectrophotometric finish at 280 nm is used for the assay with limits of 95 to 105 percent.

5.2 Identity and Color Tests

Aspirin can be identified by the following name tests:

<u>Test</u>	<u>Color</u>	<u>Ref.</u>
Trinder's reagent	Purple after hydrolysis	63
McNally's test	Red	63
Mandelin's reagent (Ammonium vanadate)	Green with blue tint; changes to red-violet	81
Feigl	Saponif. to salicylic acid. Reaction with KOH at 130°: Violet fluorescence	82
Kulberg	Saponif. to salicylic acid. Addition of FeCl ₃ in HCl: red-violet coloration	83
Vitali Morin	Orange to red	84
Kofler	Microscopic Identifi- cation	85

For identification by infrared, see Section 4.11. Identification in combination products by mass spectrometry has been described.⁸⁶

5.3 Quantitative Analysis

5.31 Elemental

The percent of carbon, hydrogen and oxygen is as follows:⁶²

	<u>% (theoretical)</u>
C ₉	60.00
H ₈	4.48
O ₄	35.53

5.32 Colorimetric

The use of basic organic dyes for ion pair extraction-photometric determination has been described.⁸⁷ After ammonia treatment, an orange-red color with CuSO₄ and H₂O₂ (Denigès) can be quantitated.⁸⁸ A water insoluble violet complex (λ max. 620 nm) with 2-picoline-Cu(II) has also been reported.⁸⁹

5.33 Ultraviolet

The maximum at 277 nm has been used to determine aspirin in tablets after chromatography (see Section 5.43). It also has been used to determine aspirin in mixtures with other drugs (cf. 21). For simultaneous determination of

aspirin and salicylic acid, see Section 5.61.

5.34 Infrared

The infrared spectrum has been used to determine aspirin in combination products. Accuracy of 1-2% has been claimed (cf. 90,91). For aspirin, the absorption maximum at 1765 cm^{-1} has been used.⁹²

5.35 Fluorescence - Phosphorescence

Although fluorescence of aspirin, as contrasted to that of salicylic acid, is weak (see Section 4.13), it has been used for the determination in tablets.²² Phosphorimetry (see Section 4.13) has been described as useful in the determination of aspirin in blood serum and plasma.²³ Since the phosphorescence of salicylic acid at the maximum of 410 nm is about 500 times weaker, it does not interfere.

5.36 Titrimetric - Electrochemical

Although theoretically aspirin could be titrated directly with alkali, this tends to give inaccurate results due to its instability in alkali and, therefore, the compendial methods back titrate after saponification (cf. 79). However, non-aqueous titration is possible and desirable, particularly for determination in combination products. Sodium methoxide in benzene-methanol is used as the titrant and methylisobutyl ketone as the solvent. The end point is determined potentiometrically.⁹³ Alternately, tetrabutyl ammonium hydroxide and DMF as titration solvent have also been used.^{94,95} Titration in ethylene diamine has also been described.⁹⁶ Potentiometric measurements of ion-pair association and selective acid strength in ethylene diamine and water has been reported.⁹⁷ Potentiometric titration in aqueous medium has also been described⁹⁸ as has colorimetric.⁹⁹

The direct current and alternating current polarographic response of aspirin in an aprotic organic solvent system (acetonitrile - 0.1M tetrabutyl ammonium perchlorate) has been studied.¹⁰⁰ The following values were obtained:

1. dc half-wave potential: $E_{1/2} = -1.64$
2. ac fundamental harmonic peak potential: $E_p = -1.76$

3. ac second harmonic minimum
potential: $E_{\min} = 1.87$

n values calculated from the three modes are: 0.44; 0.45 and 0.40. Approximate detection limits (moles/liter) for the three modes are: 5×10^{-5} ; 1×10^{-4} ; 1×10^{-4} .

On a rotating disk electrode, aspirin was reduced to the aldehyde.¹⁰¹

5.37 Miscellaneous (NMR)

Nuclear magnetic resonance spectrometry has been used to quantitate aspirin in a combination product with a coefficient of variation of 1.1.¹⁰² For quantitation, the shift at 2.3 ppm representing the ester methyl group was used.

5.4 Chromatographic Methods

5.41 Paper

Paper chromatographic systems have been tabulated in Table 4.

TABLE 4

Solvent systems:	<u>R_fA*</u>	<u>R_fS*</u>	<u>Detection</u>	<u>Ref.</u>
Pet-ether, Methanol, Benzene and Water (25:20:20:0.05)	0.05	-	FeCl ₃	103
Iso-propyl alcohol, Water, Ammonia (15:85:10)	-	-	FeCl ₃	104
Methanol, Water, Ammonia (10:90:10)	-	-	FeCl ₃	104
Butanol, 25% Ammonia (4:1)	0.45	-	U.V.	105
0.75% Nitric Acid	0.8	0.6	Fluorimetry	106

*R_fA = aspirin; R_fS = salicylic acid.

Cellulose anion-exchange paper chromatography with 5% acetic acid-n-propanol (5:1) gave good separation, detected by U.V. light, of aspirin (R_f 0.07) from salicylic acid (R_f 0.51).¹⁰⁷

5.42 Thin Layer

TLC has been used to identify and quantify aspirin in pharmaceutical preparations and body fluids. Data have been summarized in Table 5. Readout by densitometers^{108,109} and TLC separation as student experiments¹¹⁰ have also been described.

5.43 Column

As mentioned in the historical synopsis (Section 5.1), Levine¹²¹ perfected the compendial partition column procedure in which aspirin in chloroform is first trapped in an immobile phase of sodium bicarbonate on a column of siliceous earth (celite) then eluted with a solution of acetic acid in chloroform and measured spectrophotometrically. This has been also used for separation in combination products.⁸⁰ For the determination of salicylic acid in presence of aspirin by this method, see Section 5.61. Ion exchange columns filled with strongly or weakly basic anion exchange resin in the acetate or chloride cycle have also been used for separation of aspirin in combination products.^{122,123,124} This has also been adapted for a student experiment.¹²⁵ A Sephadex-G25 column has been used for the separation of aspirin from salicylic acid.¹²⁶

5.44 High Pressure Liquid

This newest of chromatographic techniques has already been used quite extensively for the determination of aspirin in pharmaceutical products. Separation on columns filled with anion-exchange resin¹²⁷ or cation-exchange resin with and without counter ions^{128,129} were investigated in detail. Silica surface columns have also been used¹³⁰ as have been reverse phase (octadecyl) ones.¹³¹⁻¹³⁴ U.V. detection was used throughout.

5.45 Gas-liquid

Determination of aspirin by gas-chromatography was first reported by Hoffman and Mitchell¹³⁵ in 1963 who separated it from other tablet ingredients by direct chromatography on tetrafluoroethylene polymer coated with Dow-Corning silicone, using a flame ionization detector and an integrator. A glass-bead column, coated with carbowax and isophthalic acid has also been used.¹³⁶ Most other investigators made the methyl- or trimethylsilyl-derivative prior to chromatography. As

TABLE 5

Thin Layer Chromatography of Aspirin

<u>Support</u>	<u>Solvent System</u>	<u>Rf.Asp.</u>	<u>Rf.Sal.</u>	<u>Detection</u>	<u>Ref.</u>
Silica	MeOH-HOAc-Et ₂ O-C ₆ H ₆ (1:18:60/120)	~0.9	-	UV	111
Silica Gel G	Et ₂ O-AcOEt (1:4)	0.26	-	-	112
Silica	C ₆ H ₆ -HOAc-MeOH-CHCl ₃ - Pet-ether	0.42	0.44	UV	113
Polyamide	CH ₃ Cl ₃ -C ₆ H ₆ -90% HCO ₂ H (5:1:0.1)	0.44	0.24	UV	114
Polyamide	iso-PrOH-H ₂ O-90% HCO ₂ H (1:5:6:01)	0.32	0.08	UV	114
Polyamide	CH ₃ Cl ₃ -90% HCO ₃ H (20:0.1)	0.47	0.24	UV	114
Polyamide	Cyclohexane-CHCl ₃ -HOAc (4:5:1)	0.61	0.41	UV	114
Silica Gel G	Cyclohexane-CHCl ₃ -HOAc (50:40:10)	0.36	-	K ₄ (Fe(CNa)) ₃	115
Polyamide	CHCl ₃ -Cyclohexane-HOAc- Dioxane (40:60:1:10)	0.48	0.14	UV	116
	CHCl ₃ -Cyclohexane-HOAc (40:60:1)	0.37	0.12	UV	116
Silica Gel G	EtOH-IsoPrOH-Xylene- CHCl ₃ (12.5:12.5:25:50)	0.04	-	Iodine	117

TABLE 5 (continued)

<u>Support</u>	<u>Solvent System</u>	<u>Rf.Asp.</u>	<u>Rf.Sal.</u>	<u>Detection</u>	<u>Ref.</u>
Silica	$\text{CHCl}_3 - (\text{CH}_3)_2\text{CO}$ (9:1)	0.075	-	NH_4 Vanadate	118
	$\text{MeOH} - \text{NH}_3$ (100:1.5)	0.75	-	NH_4 Vanadate	118
Silica Gel GF	$\text{EtOAc} - t\text{-PrOH} - \text{NH}_3$ (40:30:3)	0.25	-	UV	119
Silica Gel GF	$\text{EtOH} - \text{HOAc} - \text{H}_2\text{O}$ (60:30:10)	0.92	-	UV	119
Silica Gel GF	C_6H_6	0.10	-	UV	119
Silica Gel 60	$\text{C}_6\text{H}_6 - \text{Dioxane} - \text{HOAc}$ (60:20:2)	-	-	Fluorimetry	120

methylating agents, diazomethane in ether alcohol¹³⁷ or tetrahydrofuran,¹³⁸ methanol with boron trifluoride¹³⁹ and methyl iodide with potassium carbonate¹⁴⁰ were used. For trimethylsilylation hexamethyl disilazane,¹⁴¹⁻¹⁴³ N-O-bis(trimethylsilyl) acetamide and other trimethylsilyl donors,¹⁴⁴ BSTFA¹⁴⁵⁻¹⁴⁶ and MSTFA¹⁴⁷ have been tried. It was noted that derivatization with BSTFA or MSTFA resulted in slight hydrolysis of aspirin.¹⁴⁷

5.5 Electrophoretic Methods

Aspirin can be separated from salicylic acid by ionophoresis at a pH of 4-5.¹⁴⁸ Separation of aspirin in combination products has been achieved with paper strip electrophoresis, using buffers at pH 2-8 and a 200 V. applied potential.¹⁴⁹ Aspirin was separated from metabolites by paper electrophoresis in a phthalate buffer of pH 3.2 and an ionic strength of 0.0125-0.0500.¹⁵⁰

5.6 Determination of Impurities

5.61 Salicylic Acid

As has already been pointed out, the determination of salicylic acid is intricately interwoven with that of aspirin itself. There is the convenient color reaction with ferric chloride which was already used by Dreser¹ to determine free salicylic acid in his own urine after the ingestion of aspirin. However, this reaction is not too specific and considerable work has gone in the development of interference free methods.

My task has been made easy since there is an excellent review by C.A. Kelly¹⁵¹ on the determination of salicylic acid in aspirin and aspirin products. A more recent review has been compiled by S.L. Ali.¹⁵²

I, therefore, have restricted my tabulation of methods to those references which discuss determination of salicylic acid in biological specimens, which were not covered by Kelly, to important references which have been published since the Kelly review and references which are pertinent to other sections of this profile.

References for the determination of salicylic acid:

1. Colorimetric (iron complex): 77,78,153,154
(Folin-Ciocalteu reagent): 218,219

2. Nonaqueous titration: 104
3. Iodometric: 131
4. Spectrophotometric: 20, 156-158
5. Fluorometric: 22, 159, 160
6. Infrared: 92
7. Column: 57, 137, 138, 103
8. PC (for R_f values and solvent systems see Section 5.41): 106, 107
9. TLC (for R_f values and solvent systems see Section 5.42): 114, 116, 119
10. VPC: 106, 107, 140, 141, 144, 145, 146, 147
11. HPLC: 152, 164
12. Automated: 165-167

5.62 Acetic Acid

It is easily forgotten that aspirin degrades to acetic as well as salicylic acid. And, indeed, any smell aspirin might have is due to acetic acid. However, the volatility of acetic acid does not make the determination of acetic acid a reliable tool to measure stability or degradation.

A.N. Smith in 1920¹⁶⁸ devised a method to determine acetic acid in aspirin by bubbling dry air through a thin layer of powdered aspirin, trapping the acetic acid in water and back-titrating it with alkali. Gas chromatography¹⁶⁹ has also been used.

5.63 Acetylsalicylic Anhydride and Acetylsalicylsalicylic Acid

In addition to salicylic and acetic acids, very small quantities of acetylsalicylanhydride (0.0012 to 0.024%) and acetylsalicylsalicylic acid (0.03 to 0.1%) have been found in aspirin preparations. The former has been determined by gas chromatography,¹⁴⁰ TLC¹⁷⁰ and spectrophotometry,¹⁷¹ the latter by gas chromatography.^{140, 144} A controversy is still ongoing (cf. 152) whether the occasionally observed hypersensitivity against aspirin is caused by these two impurities and whether the basis of the adverse reaction is immunological.

6. Stability - Degradation

That aspirin is sensitive to moisture is well-known and most of us at one time or another have observed the growth of salicylic acid whiskers on aspirin tablets left for too long in a humid bath-

room wall cabinet. The hydrolysis of aspirin to salicylic acid was already of concern to the pharmacologist, Dreser¹ (see Section 1.2), who in 1899 prepared what nowadays one would call a preformulation profile. He tested the hydrolysis of aspirin in acid and alkaline solutions and, by perusing textbooks of Ostwald and Nernst to devise the proper equations, determined hydrolysis constants of 3.3×10^{-4} at "body temperature" in acid medium and 2.5×10^{-2} at room temperature in alkaline medium. He also observed that aspirin was easily split in weakly alkaline medium which had consequences for the metabolism (see Section 7). One could say that he established the first profile for the pH dependence of hydrolysis of aspirin. Of course, the definition of pH was unknown at the time.

The next systematic study of the hydrolysis of aspirin in water, albeit at 100° C., was undertaken by Rath¹⁷² who determined a hydrolysis constant of about 0.17 depending on experimental conditions. Much work has been done since, and it is quite evident that this seemingly simple hydrolysis to acetic and salicylic acids is both complex and controversial. I am fortunate that I can refer the reader to the excellent and detailed review by Clark A. Kelly¹⁵¹ already mentioned in Section 5.61. The most complete and thorough kinetic studies of the factors involved in the hydrolysis of aspirin are undoubtedly those by Edwards¹⁷³, which were further elaborated by Garrett.¹⁷⁴

Stability and decomposition kinetics of aspirin both as a solid and in solution continue to be studied. The topochemical decomposition pattern of aspirin tablets has been explored.¹⁷⁵ The degradation of aspirin in the presence of sodium carbonate and high humidity was studied by x-ray diffraction.¹⁷⁶ The activation energy of decomposition by water vapor in the solid state was found to be 30 kcal/mol.¹⁷⁷ The effect of common tablet excipients on aspirin in aqueous suspension was also studied.¹⁷⁸

An exhaustive study of the stability of aspirin in polyethylene glycols (substituted, unsubstituted and esterified), as well as other polyhydric alcohols, was undertaken by Whithworth and collaborators.¹⁷⁹⁻¹⁸³

It was found that decomposition is in part due to transesterification and that substitution increases stability. The effect of gamma radiation on aspirin has been described.¹⁸⁴ The pH stability profile of aspirin according to Edwards has been made the subject of a student experiment.¹⁸⁵

7. Pharmacokinetics - Drug Metabolic Products

When Dreser¹ investigated the pharmacological properties of aspirin in 1897 (see Section 1.2), he postulated that, based on the easy hydrolysis of aspirin in weakly alkaline medium, aspirin also should be converted to salicylic acid in vivo. And, indeed, he found that only 22 minutes after ingestion of aspirin, his own urine gave a positive reaction for salicylic acid with ferric chloride. After 12 hours, he could no longer detect salicylic acid in his urine. He found no evidence for aspirin itself in urine, nor did he find a combination of aspirin with glycine analogous to the formation of hippuric acid already known at the time. He had evidence for formation of another nitrogen containing derivative. This early investigation should give food for thought to those who believe that pro-drugs and pharmacokinetics are recent discoveries.

In 1911, Neuberg¹⁸⁶ detected small amounts of gentisic acid (2,5-dihydroxybenzoic acid) in the urine of dogs dosed with aspirin. The metabolism of aspirin is intertwined with that of salicylic acid, but I was unable to ascertain who first reported the metabolic formation of salicyluric acid, the major metabolite of both salicylic acid and aspirin, specifically after administration of aspirin.

Reviews by Puetter¹⁸⁷ and by Levy,¹⁸⁸⁻¹⁹⁰ taken together present a comprehensive picture of the pharmacokinetics and metabolism of aspirin.

Once absorbed, aspirin is rapidly converted to salicylic acid. After i.v. administration, the half life of aspirin in the human organism was found to be only 15 minutes¹⁹¹ by Rowland and Riegelman who also estimated that only 20% of the in vivo hydrolysis takes place in blood.¹⁹²

Apparently, all tissues investigated possess

esterases which can split aspirin; however, most of it seems to be hydrolyzed in the liver.¹⁸⁷ While the major pathway of hydrolysis leads to free acetic acid, it is noteworthy that a significant portion of the acetic acid is not set free but used for transacetylation of certain factors which play a role in the inhibition of platelet aggregation¹⁹³ only shown by aspirin but not by salicylic acid. The kinetics of this important pathway still need exploration.¹⁸⁷

Aspirin serum esterase activity is species dependent.¹⁹⁴ In man it is sex linked since it was found to be higher in men than women.¹⁹⁵ It also seems to be age dependent.¹⁹⁶

Once aspirin is hydrolyzed to salicylic acid, it follows the metabolic pathway of the latter. The metabolic products of salicylic acid are presented in Figure 9. Over the last decade, the pharmacokinetics of salicylates in the healthy and diseased organism have been explored in great detail by Levy.¹⁸⁸ Salicylic acid (II) is eliminated by five parallel and competing pathways (Fig. 9) leading to renal excretion. These are: excretion unchanged (II), conjugation with glycine to form salicyluric acid (III) -- the major pathway -- conjugation of the carboxyl or phenolic hydroxyl group to form glucuronides IV and V and hydroxylation to gentisic acid (VI). To a minor degree, salicyluric acid can be further conjugated with glucuronic and sulfuric acids.¹⁸⁷ The major metabolic pathways (salicyluric and salicyl phenol glucuronide) are easily saturated in the usual dosage range for treatment of inflammation.¹⁹⁷ For the clinical implications of these non-linear pharmacokinetic characteristics, I refer to the reviews by Levy.¹⁸⁸⁻¹⁹⁰

To properly treat bovine "sufferers of headaches" (inflammation) the pharmacokinetics of aspirin in cattle have also been explored.¹⁹⁸

8. Bioavailability - Dissolution

In the last two decades, the concept of bioavailability has gained prominence and with it dissolution as a possible in vitro model for drug absorption. In 1960, Swintosky and Blythe¹⁹⁹

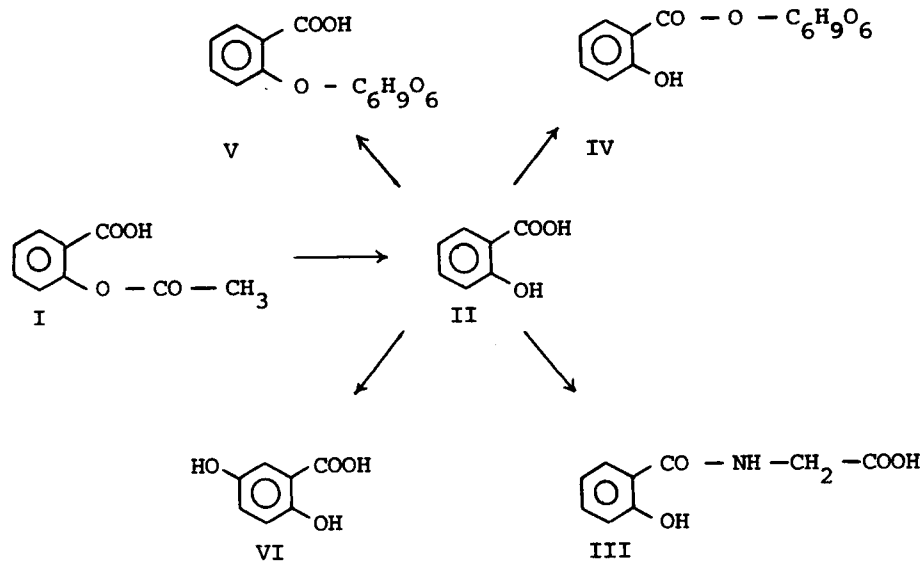


Figure 9. Drug Metabolic Products of Aspirin

compared the relative availability of aspirin from enteric coated and compressed tablets by measuring the excretion of salicylate. About the same time, Levy^{200,201} started to compare dissolution and absorption rates of different commercial aspirin tablets, found good correlation and proposed that the U.S.P. tablet disintegration test be replaced by a dissolution test; a suggestion which as of this writing has not been heeded. These studies were extended and further perfected by Gibaldi.¹⁶³ Many other papers on this subject have appeared. Particularly, the influence of the crystal habitat (see Section 4.243) on absorption has been studied (cf. 202). Significant intraindividual, but not interindividual, variations in blood plasma aspirin levels were found in young male subjects after administration of aspirin as tablets or solutions. The plasma level curves obtained for tablets were more variable than those obtained for solutions.²⁰³ Levy²⁰⁴ found that using urinary excretion measurements for evaluation of aspirin dosage forms, with different absorption rates in man, requires that such measurements be made during the first hour after drug administration. The use of a pH-stat for testing dissolution of various aspirin dosage forms has been described.²⁰⁵

9. Determination in Biological Fluids and Tissues

All the advances in pharmacokinetics and drug metabolism described in Sections 7 and 8 would not have been possible without the availability of the proper analytical methods. The following is a tabulation of publications in this field, most of which have already been discussed in Section 5. It should be mentioned that a few publications talk about aspirin blood levels, but really mean salicylate levels. The following tabulation covers only those papers where aspirin was differentiated from other salicylates by chromatography or other means. It seems that the "workhorse" for serum salicylate levels is still the colorimetric (ferric-nitrate) method of Brodie, Udenfriend and Coburn¹⁵³ published in 1944, or modifications thereof. Simplified versions (cf. 206) may lead to erroneous results under certain conditions.²⁰⁷ The method is also applicable for urinary metabolites after proper hydrolysis (cf. 208). For other methods restricted to salicylic acid, see Section 5.61. The first gas chromatographic separation of aspirin

and salicylic acid in plasma was described by Rowland and Riegelman,¹⁴¹ who presented a brief review of earlier methods.

Methods for aspirin:

Colorimetric (with differential extraction): 218, 219

Spectrophotometric (differential): 20, 209

Fluorometric (as salicylic acid): 160, 210

Phosphorimetric: 23

VPC: 137, 141, 143, 145, 146

PC: 106, 107, 119

TLC: 118, 211

Mass Spectrometric: 37

Radioisotopic: 212-214

10. Determination in Pharmaceutical Preparations

The following tabulation of references highlights those methods (see Section 5) useful in pharmaceutical analysis.

1. Determination in tablets:

Differential U.V.:	157, 158
Automation:	166
Fluorometric:	22
Column:	121
TLC:	120
GC:	136, 139, 144

2. Determination in buffered tablets:

Fluorometric:	159
Column:	161, 162

3. Determination in combination products:

a. General Identity tests: 83, 86, 103, 104, 105, 112

b. Aspirin, caffeine, acetophenetidine
(and more components)

UV:	21, 215
IR:	91-93
NMR:	102
Electrophoresis:	149
TLC:	110, 111
Column:	80
Ion-exchange column:	123
HPLC:	127, 130, 131
VPC:	135, 142
Pot. titration:	216

- c. Aspirin, acetaminophen, caffeine:
Nonaqueous titration: 94,217
Potentiometric: 102
- d. Aspirin-barbiturate combinations:
Nonaqueous titration: 93
- e. Cough-cold mixtures:
HPLC: 132

11. Acknowledgments

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The literature has been covered systematically through Chemical Abstracts, Volume 88 (1978), with a few stray references beyond.

BROMOCRIPTINE METHANESULPHONATE

Danielle A. Giron-Forest and W. Dieter Schönleber

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1. Introduction

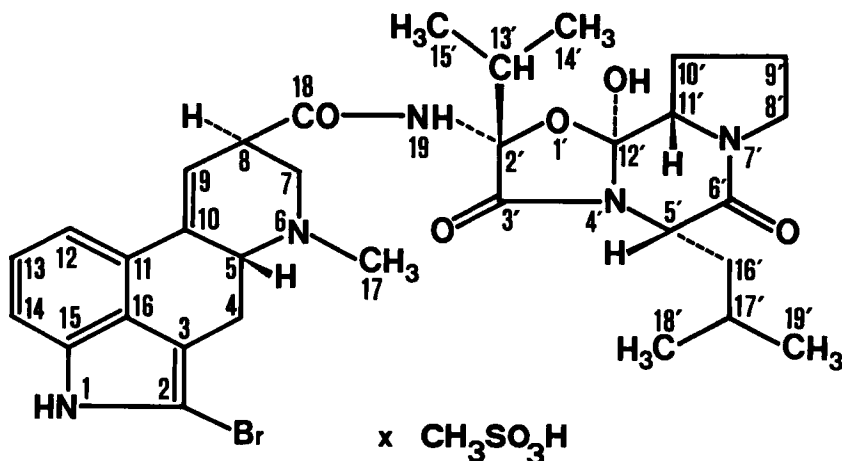
1.1 History

The most valuable pharmacological properties of the peptide type ergot alkaloids induced a variety of attempts of chemical derivatisation of the parent compounds (1,2 + lit. quoted therein). In one of these the bromination of α -ergocryptine led to a product of highly interesting pharmacology, namely bromocriptine.

It became known to suppress prolactine secretion, and it is therefore a useful tool in the treatment of prolactine dependent disorders, such as galactorrhoea associated with hyperprolactinemia and postpartum, as well as certain kinds of sterility (3 - 9). In more elevated doses, the drug is a potent antiparkinsonicum. In addition, there is recent evidence of bromocriptine playing an important role in the trace heavy metals balance of the brain (10).

1.2 Name, Formula, Molecular Weight

Bromocriptine mesilate is 2-bromo- α -ergocryptine methane-sulphonate or 2-bromo-12'-hydroxy-2'-(1-methylethyl)-5'-(2-methylpropyl-5'- α -ergotaman-3',6',18-trione methane-sulphonate or Bromocriptinum (INN). It is the active ingredient in Parlodel® dosage forms.



Chemical Abstracts Registry Numbers :

25614-03-3
(26409-15-4)
(47830-26-2)

Average Molecular Weights :

base : 654.61
mesilate: 750.71

Average Formulas :

base : $C_{32}H_{40}BrN_5O_5$
mesilate: $C_{33}H_{44}BrN_5O_8S$

1.3. Appearance, Colour, Odour

Grey tinged white or light yellow, finely crystalline powder, odourless or of weak, characteristic odour.

2. Physicochemical Properties

2.1 Elemental Analysis

The elemental analysis of bromocriptine mesilate yielded the following results (11):

element	% calculated	% found
C	52.8	53.2
H	5.9	6.0
Br	10.6	10.5
N	9.3	9.2
O	17.0	16.8
S	4.3	4.4

2.2 Spectra

2.21 Infrared

The infrared spectrum of bromocriptine mesilate in a KBr pellet is given in fig. 1. It was recorded on a Perkin Elmer 257 infrared spectrophotometer.

The main characteristic bands are the following :

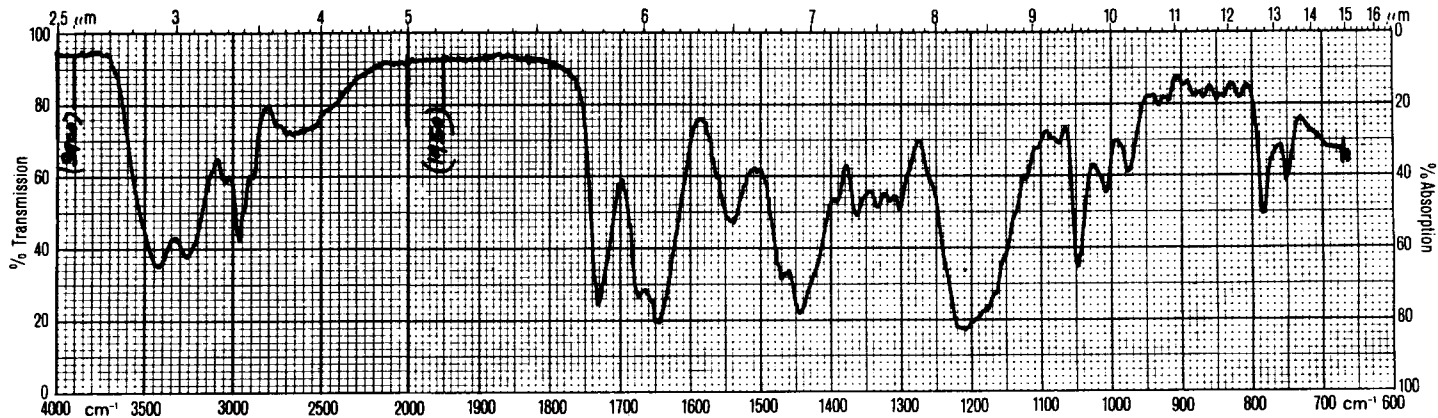


Figure 1. Infrared Spectrum of Bromocriptine Mesilate
in a KBr Pellet.
Instrument: Perkin Elmer 257

wave number (cm^{-1})	assignment
3200 - 2900	C-H-stretching vibrations
1728	C=O-stretching of 5-membered ring lactam (cyclo1)
1642, 1672	C=O-stretching of 6-membered ring lactam (amide I)
1560	amide-II band and C=C-stretching

The spectrum of the base has been reported by P.A. Stadler and co-workers (11).

2.22 Ultraviolet

The ultraviolet spectrum of bromocriptine mesilate was recorded on a Philips 1700 uv spectrophotometer in 0.1 M methanolic methanesulphonic acid solution. It is given in fig. 2. A maximum occurs at about 308 nm with a log molar absorptivity of 4.0. In 1:1 dichloromethane/methanol solution the absorption maximum was reported as 306 nm log = 3.988 (11).

2.23 Fluorescence

Bromocriptine mesilate exhibits fluorescence like the other lysergic and isolysergic acid type alkaloids. In 2 percent ethanolic tartaric acid solution, the emission maximum appears at 402 nm (excitation at 325 nm). See fig. 3.

2.24 Proton Nuclear Magnetic Resonance

The PMR spectrum of bromocriptine mesilate in deuterated dimethyl sulphoxide as obtained on a Bruker HX-90 NMR spectrometer is presented in fig. 4. TMS served as internal standard. The characteristics of the spectrum are given in the following table (see also 12, 13 and lit. quoted therein):

Chemical Shift downfield (ppm)	PMR-spectrum and Assignment		
	Intensity	Multiplicity	Assignment
11.85	1 H	Singlet	indole-NH
10.45	1 H	Singlet	NH ⁺
9.55	1 H	Singlet	CONH (18-19)
7 - 7.3	4 H	Multiplet	H-C ₁₂ , C ₁₃ , C ₁₄ and OH



Figure 2. Ultraviolet Spectrum of Bromocriptine Mesilate
in 0.1 M Methanolic Methanesulphonic Acid.
 $C_A = 0.05$ mg/ml, $C_B = 0.012$ mg/ml.
Instrument: Philips SP 1700

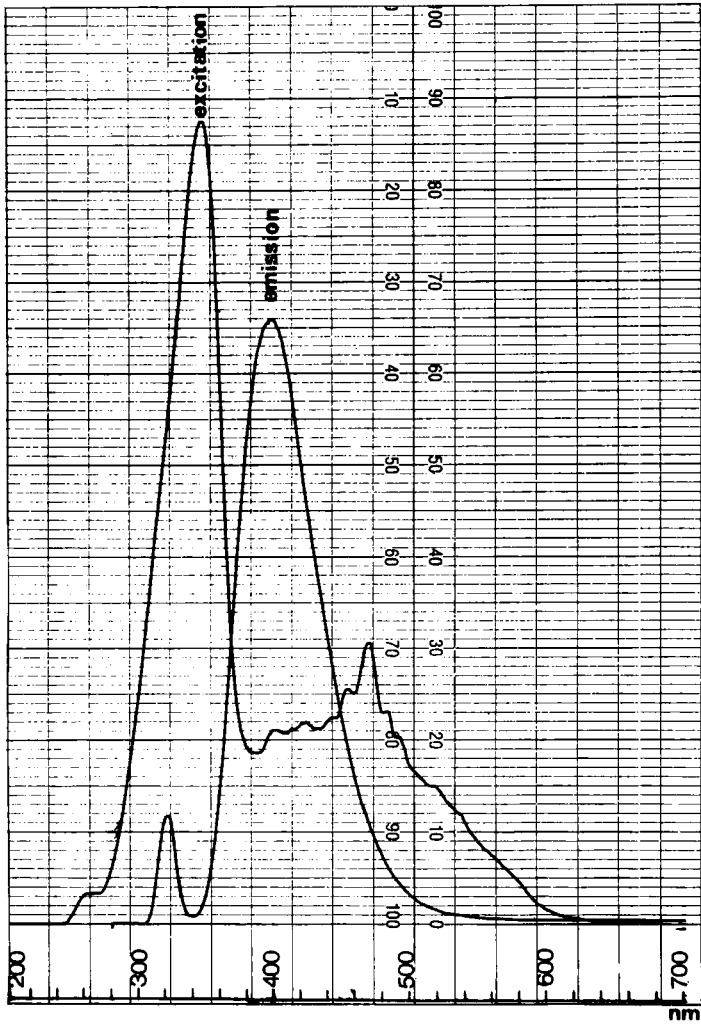


Figure 3. Fluorescence Spectrum of Bromocriptine Mesilate in 2 % Ethanolic Tartaric Acid.
C = 65 μ g/ml.
Instrument: Perkin Elmer MPF-3

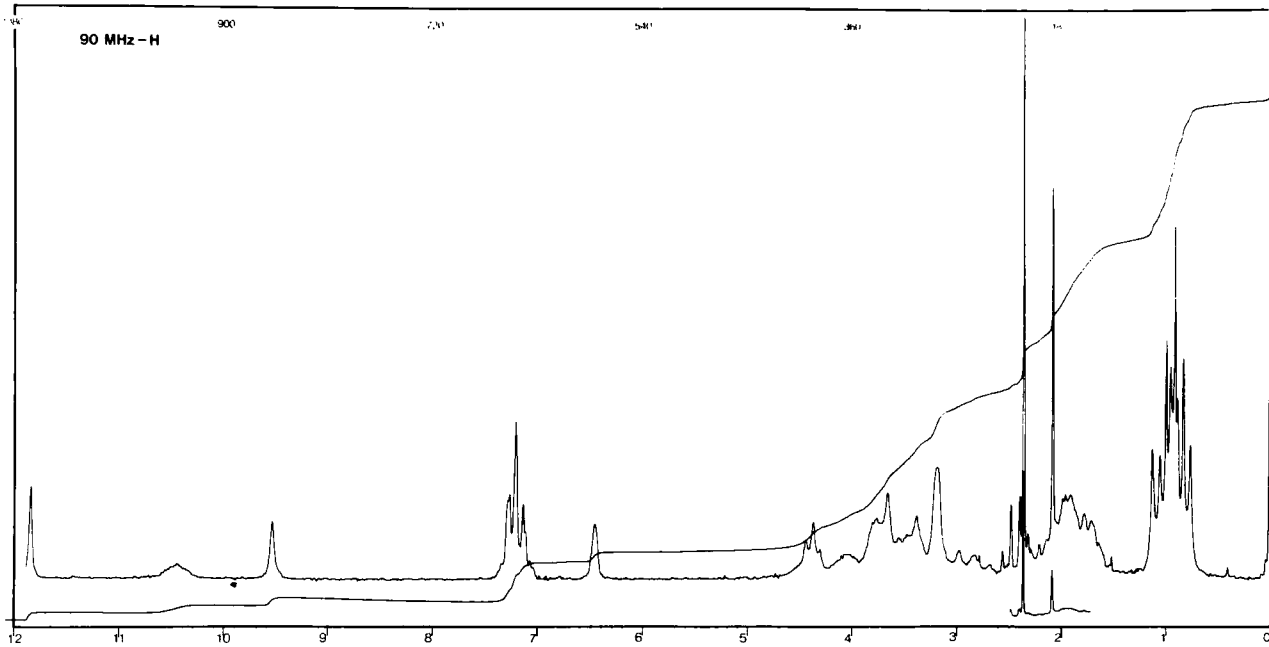


Figure 4. PMR Spectrum of Bromocriptine Mesilate in $(\text{CD}_3)_2\text{SO}$,
Instrument: Bruker HX-90 E

6.47	1 H	Singlet	$\underline{\text{H}}\text{-C}_9$
4.3 - 4.5	16 H	Triplet	$\underline{\text{H}}\text{-C}_5$,
3.2		Singlet	6-CH_3 (C-17)
3 - 4.2		Multiplet	$\underline{\text{H}}\text{-C}_8$, $\underline{\text{H}}\text{-C}_{11}$, $\underline{\text{H}}\text{-C}_4$; $\underline{\text{H}}\text{-C}_5$; $\underline{\text{H}}\text{-C}_7$; $\underline{\text{H}}\text{-C}_8$ $\text{CH}_3\text{COCH}_2\text{CH}_3$; H_2O (recryst. solvent)
2.37	3 H	Singlet	$\text{CH}_3\text{-SO}_3\text{H}$
2.1	8-9 H	Singlet	$\text{CH}_3\text{COCH}_2\text{CH}_3$
2.1 - 1.5		Multiplet	$5'\text{-CH}_2$; $5'\text{-CH}$; $2'\text{-CH}$; $\underline{\text{H}}\text{-C}_9$, ; $\underline{\text{H}}\text{-C}_{10}$,
0.75-1.15	12 H	Multiplet	$2'\text{-CH}_3$; $5'\text{-CH}_3$ and $\text{CH}_3\text{COCH}_2\text{CH}_3$

2.25 Carbon-13 Magnetic Resonance Spectrum

The spectrum of bromocriptine mesilate has been recorded in dimethyl sulphoxide using a Bruker HX-90 NMR spectrophotometer (fig. 5). The assignment of the individual signals is given in fig. 6.

2.26 Mass Spectrum

The low resolution electron impact mass spectral pattern of bromocriptine mesilate (fig. 7) corresponds quite nicely to those of the other non- and dihydrogenated ergot alkaloids (12, 13 and lit. quoted herein).

The molecular peak M of the base shows up at 653 and 655, respectively, reflecting the natural abundance of the bromine isotopes, and M-18 at 635/637 mass units. As in the parent compounds the next smaller fragment appears at M-228, indicating a substantial loss of the peptide section constituents with the tentative fragment structure F1. From this species the isopropyl group splits off easily to yield the line pair at 382/384 mass units. Loss of the entire peptide moiety leads to the formation of 2-bromo-lysergamide F2 at 345/347 m.u., the mass spectral behaviour of which corresponds to that of the bromine-free compound. Thus, it gives rise to peak pairs at 300/302 and 299/301, respectively, presumably by loss of formamide or by the rupture of the tetra-hydro-pyridine ring,

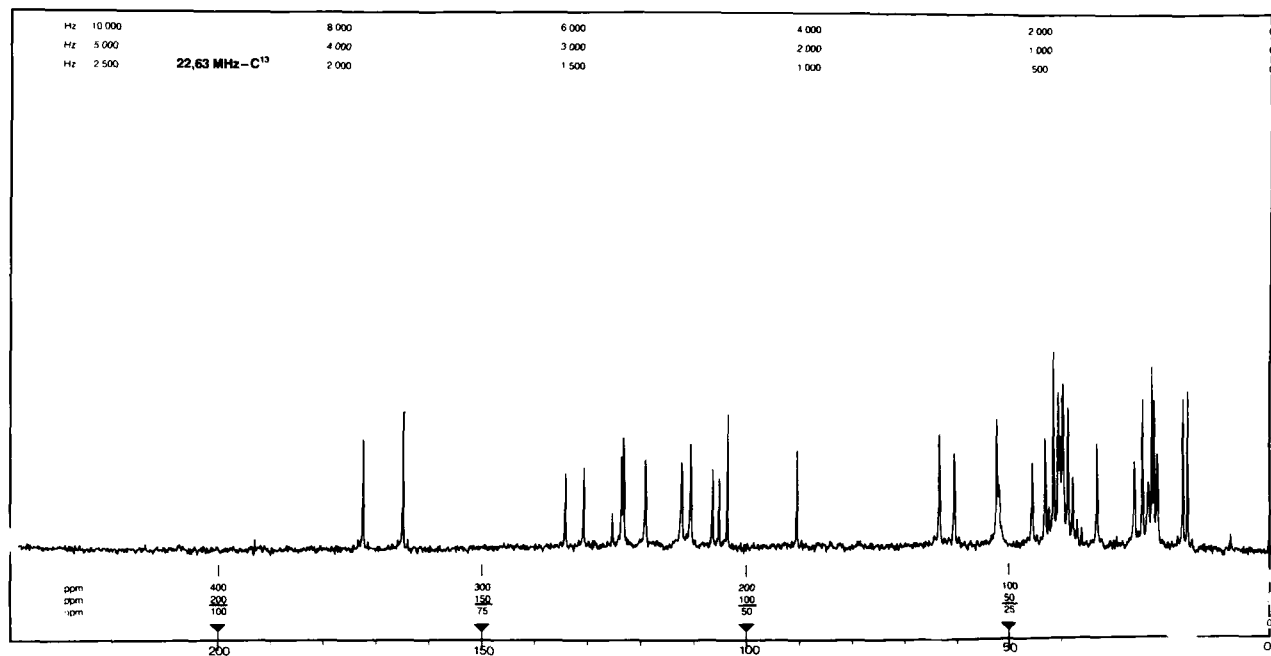


Figure 5. ^{13}C -NMR Spectrum of Bromocriptine Mesilate in $(\text{CD}_3)_2\text{SO}$,
Instrument: Bruker HX-90 E

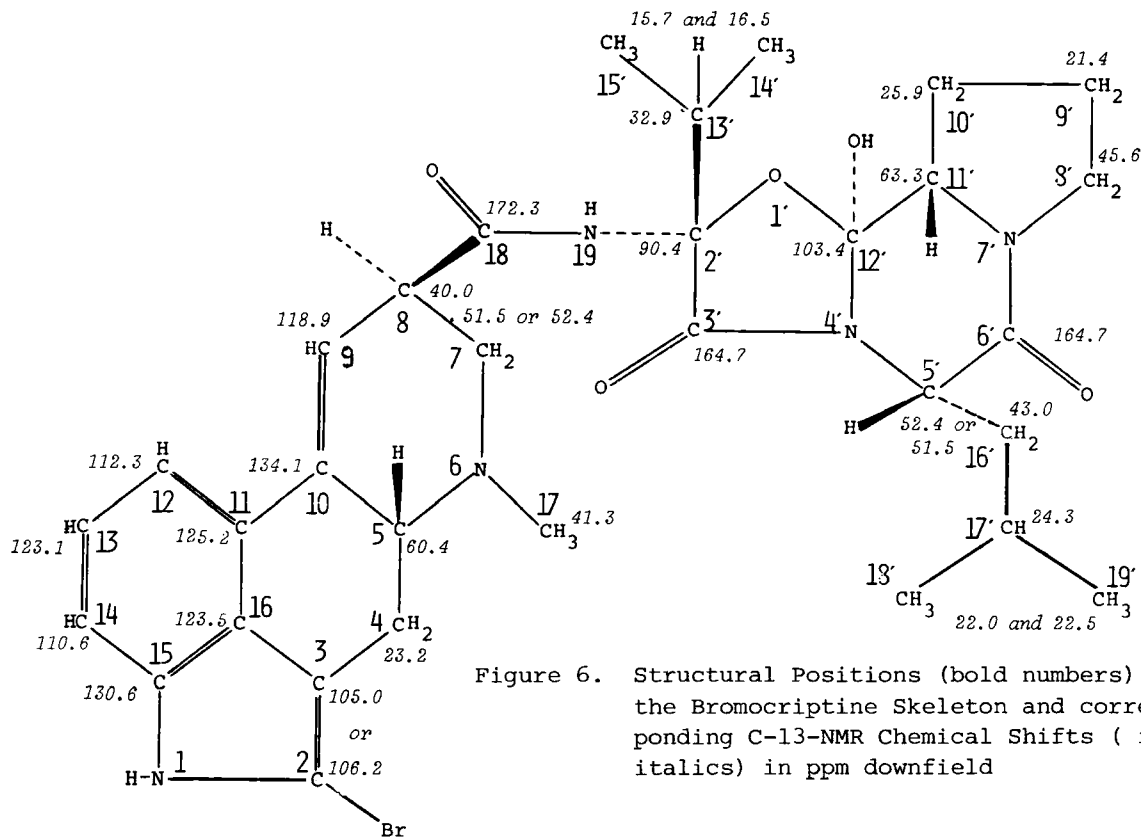


Figure 6. Structural Positions (bold numbers) in the Bromocriptine Skeleton and corresponding ^{13}C -NMR Chemical Shifts (in italics) in ppm downfield

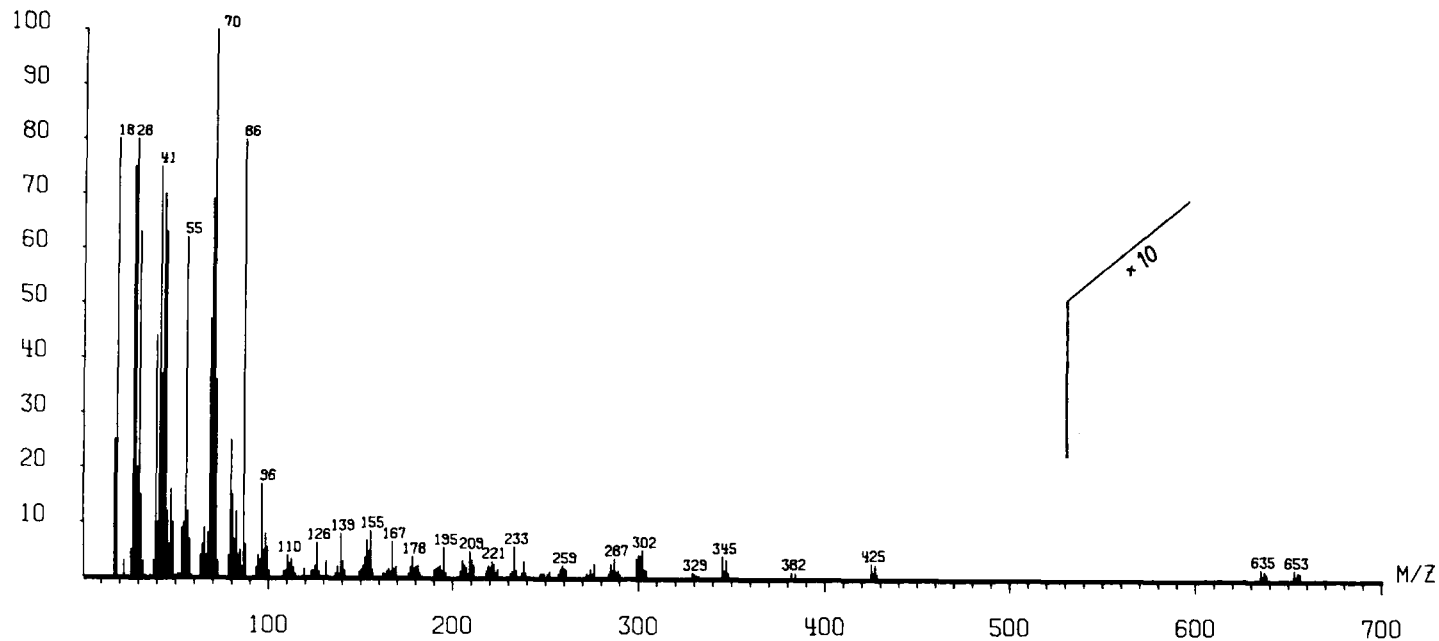
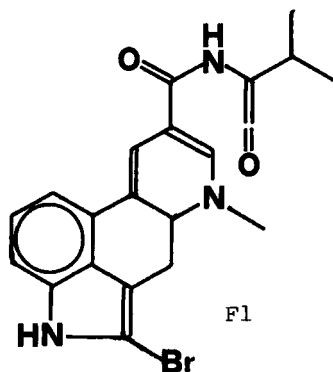
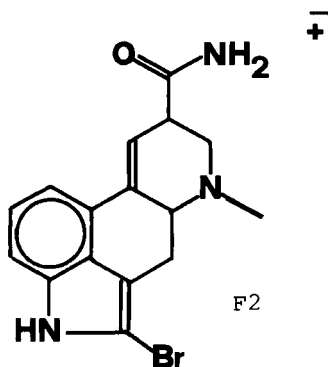


Figure 7. Low Resolution Electron Impact Mass Spectrum of
Bromocriptine Mesilate
Instrument: CEC 21-110B; Energy 70 eV, Ion Source
Temperature 160 - 200° C

a decay mode that was proposed earlier (14,15) for lysergic acid derivatives. Peak doublets at 285/287 and 274/276 m.u. indicate fragments of illicit structure still containing bromine.

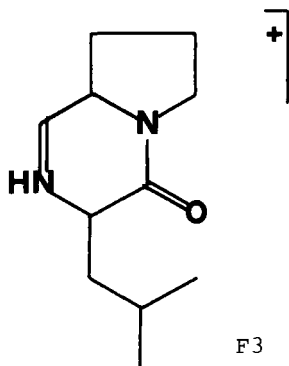


425/427 m.u.

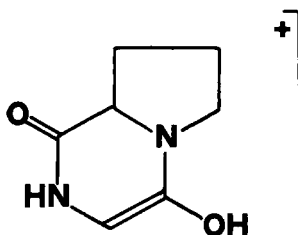


345/347 m.u.

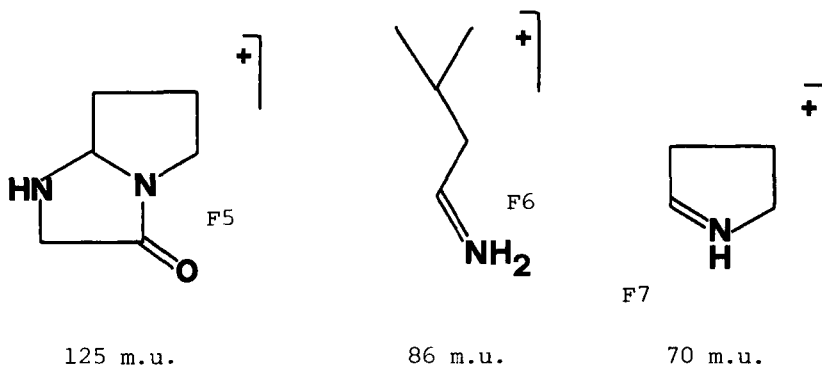
The peptide moiety itself (308 m.u.) obviously does not appear in the spectrum, whereas its fragmentation pattern with peaks at m/e 195 (F3), 167 (F3-CO), 155 - 153 (F4), 125 (F5), 86 (F6), and base peak 70 (F7) mass units, is clearly understood (14,15). The mesilate shows a signal at m/e 96.



195 m.u.



154 m.u.



2.3 Crystal Properties

2.31 Melting Characteristics

Bromocriptine mesilate decomposes between 180 and 200 °C, thus a melting point or melting range cannot be given.

2.32 Polymorphism

Investigations for the occurrence of polymorphism have been undertaken by ir spectroscopy, differential scanning calorimetry and x-ray powder diffraction (Guinier-de Wolff). No polymorphism has been observed so far. An amorphous form may be prepared artificially by rapid evaporation of a methanolic solution of the drug substance.

2.33 X-Ray Diffraction

X-ray structural analysis of bromocriptine, crystallized as the base from dichloromethane/diethylether, has been carried out on a CAD 4-diffractometer with CuK α -radiation (16). 3371 reflexions were within $\sin \Theta/\lambda < 0.62 \text{ \AA}^{-1}$. Assessment of the structure was achieved by computation to a refinement of $R = 0.033$ for the absolute configuration. The crystal data were: Space group $P 2_1$, $a = 10.681$, $b = 13.454$, $c = 11.049$, $\beta = 99.75^\circ$, $Z = 2$. In fig. 8 the resultant conformation of bromocriptine base is depicted.

2.34 Differential Scanning Calorimetry

The DSC thermogram of bromocriptine mesilate, obtained with a Perkin Elmer DSC-2 instrument at a heating rate of 20 °C/min. and in a nitrogen atmosphere, is shown in fig. 9.

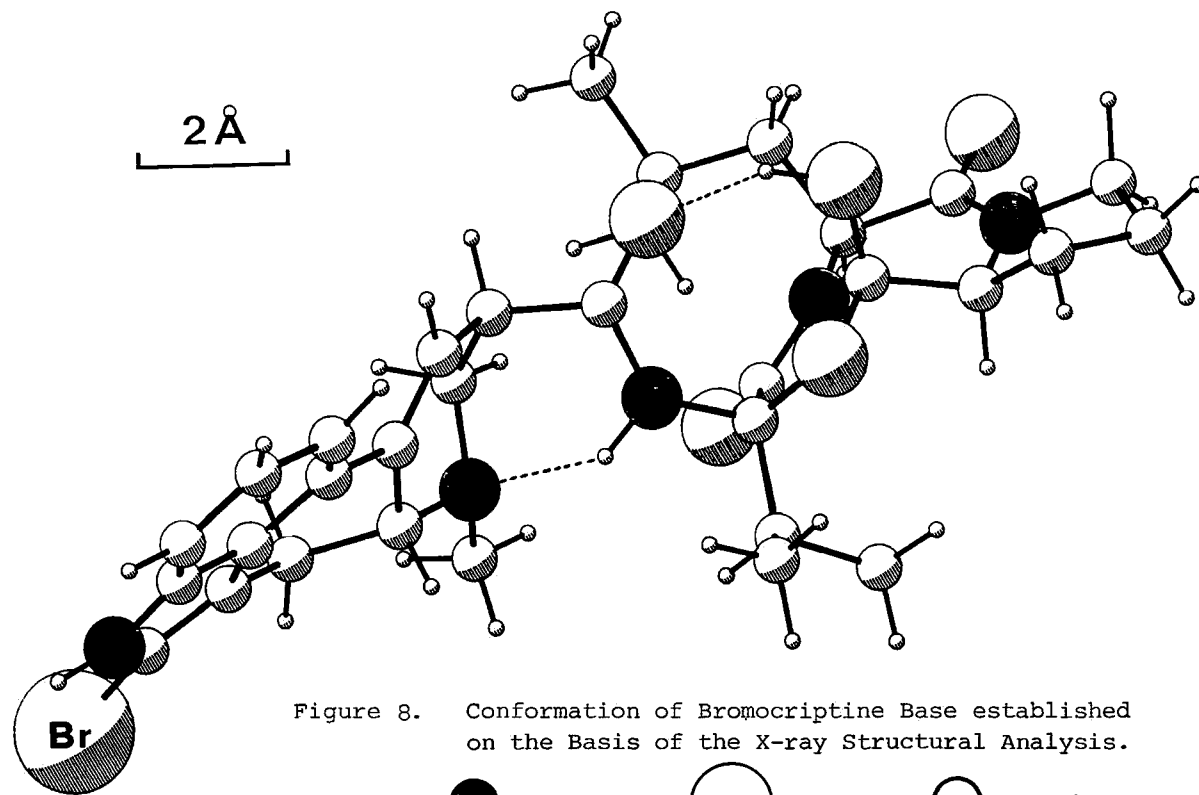


Figure 8. Conformation of Bromocriptine Base established on the Basis of the X-ray Structural Analysis.

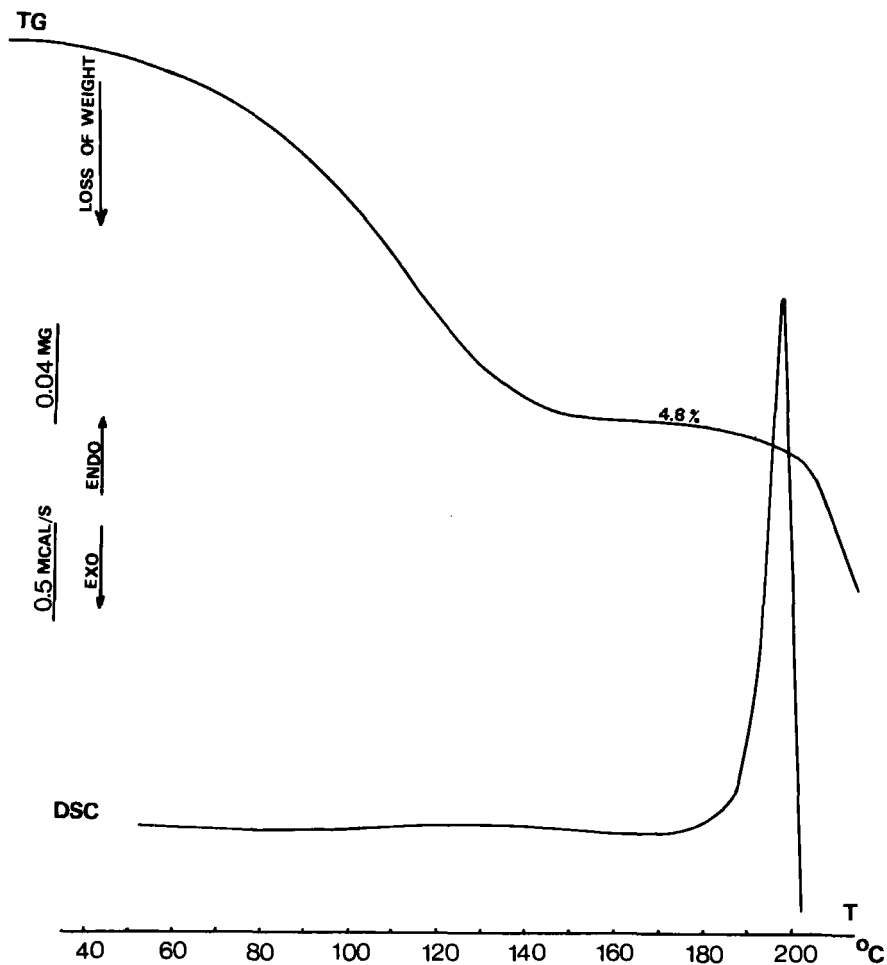


Figure 9. Differential Scanning Calorimetry and Thermogravimetry Curves of Bromocriptine Mesilate, each at a heating rate of 20°C/min. and a Nitrogen Flow of 15 ml/min.
Instruments: Perkin Elmer DSC-2
Perkin Elmer TGS-1

The melting endotherm is followed immediately by a strong exothermic degradation. Since bromocriptine mesilate decomposes under melting, the transition temperature is strongly dependent on the heating rate. A broad but weak endotherm between 40 and 100 °C indicates the volatilization of sorbed recrystallization solvent (usually butanone-2, see section 3).

2.35 Thermogravimetry

The thermogram of bromocriptine mesilate, carried out on a Perkin Elmer TGS-1 thermobalance, is given in fig. 9. The sample temperature was raised at a rate of 20°C/minute maintaining a nitrogen atmosphere. A considerable loss of weight, attributed to a loss of sorbed solvent (s. above), is observed below 130 °C. Sample decomposition obviously starts after melting.

2.4. Solubility

The solubility of bromocriptine mesilate was determined in a variety of solvents equilibrated by vibration during 24 hours at 25 °C. They are as follows:

Solvent	Solubility in mg/g	Solubility in g/100 ml
water	0.8	0.08
methanol	910	72
ethanol	23.0	1.8
2-propanol	1.2	0.1
acetonitrile	1.6	0.12
acetone	0.2	0.015
ethyl acetate	0.2	0.015
chloroform	0.45	0.06
benzene	<0.1	<0.02
hexane	<<0.1	<<0.01

At 22 ± 2 °C bromocriptine mesilate dissolves readily (>2 %) in propylene glycol, 50 % ethanol, 95 % ethanol and n-octanol, whereas it is poorly soluble (<0.1 %) in simulated gastric and intestinal fluids at 37 ± 2 °C.

2.5 Dissociation Constant

Due to the low solubility of bromocriptine mesilate in water, the pK_a value had to be determined in methyl cello-solve/water 8:2 (w/w). Titration at ambient temperature yielded pK_a as 4.90 ± 0.05 for a 0.0078 M solution.

2.6 Partition Coefficients

The partition coefficients of bromocriptine mesilate between water of pH 1.2 and n-octanol on the one hand, and water of pH 7.5 and n-octanol on the other, have been determined at 37.0 ± 0.5 °C.

water pH 1.2/n-octanol	1 : 90
water pH 7.5/n-octanol	1 : 235

2.7 Optical Rotation

Theoretically, with 6 chiral centers within the molecule (at 6, 9, 2', 5', 11' and 12' positions) 64 diastereomeric forms are possible. However, bromocriptine is sterically well defined at all of these positions, as it is derived from the naturally occurring α -ergocryptine.

The specific optical rotation of bromocriptine mesilate in different solvents is given below for 20° C corrected for loss on drying. A Perkin Elmer polarimeter 241 was used, the actual concentration being 10 mg/ml.

solvent	wavelength (nm)				
	589	578	546	436	365
specific optical rotation in degrees					
dichloromethane/					
methanol 1:1	101.0	107.5	130.1	327.8	329.4
ethanol	100.6	107.0	129.6	328.7	331.3
dimethyl formamide	127.4	135.2	162.0	388.0	392.7

For the specific rotation of 2-bromo- α -ergocryptine and -inine bases see (11).

3. Synthesis

Bromocriptine base is manufactured by bromination of α -ergocryptine with N-bromosuccinimide in dioxane solution. The mesilate is then formed by addition of methanesulphonic acid. The salt is recrystallized from butanone-2 (s. fig. 10) (2,3,11).

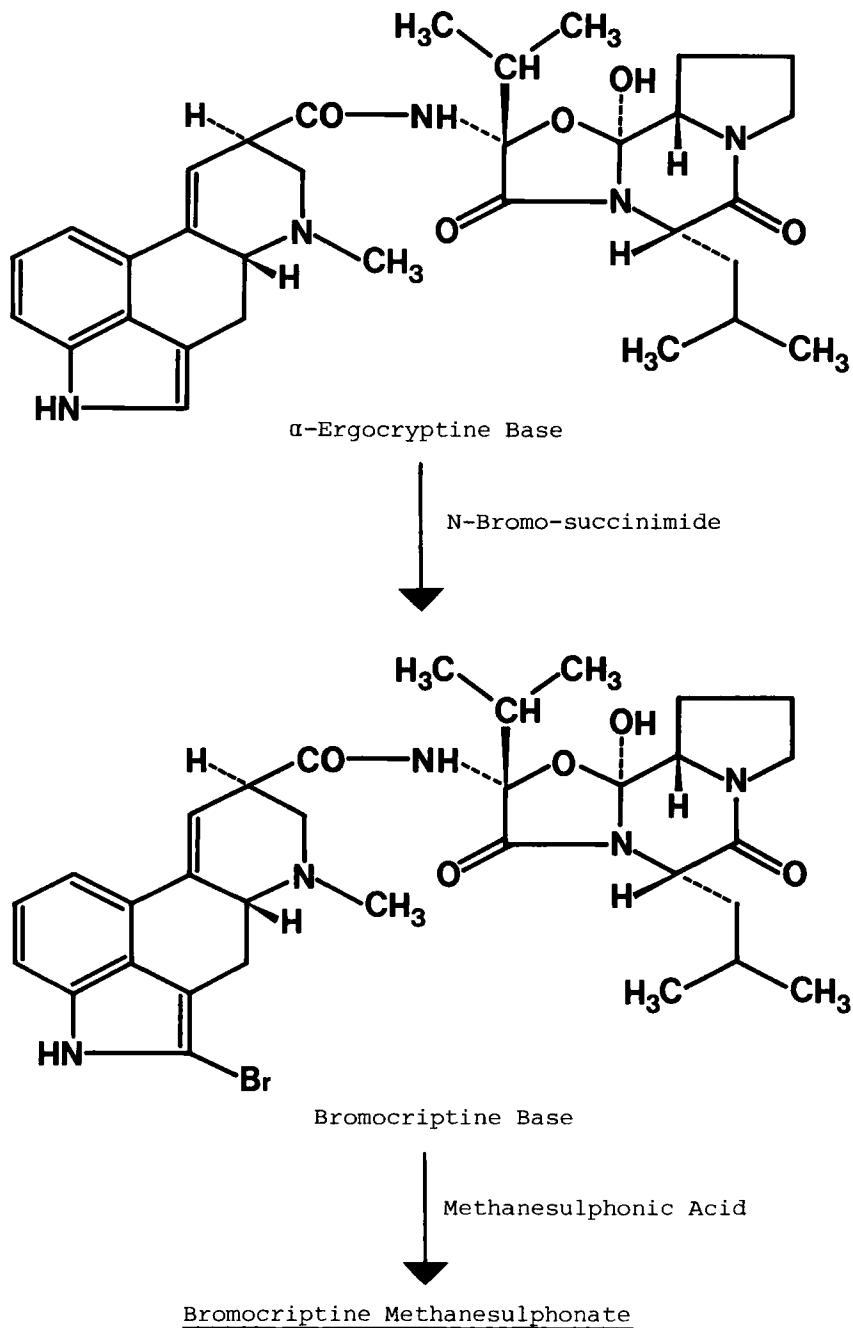


Figure 10. Route of Synthesis

4. Stability and Degradation Processes

Possible degradation pathways of ergot alkaloids at the example of ergotamine tartrate have been clearly summarized by H. Bethke et al. (17) and B. Kreilgaard (13).

As a nonhydrogenated ergot alkaloid, bromocriptine is relatively sensitive to autoxidation both in solid and in dissolved states, but degradation products have not yet been elucidated. Similarity to the oxidative transformation of the parent compounds is to be strongly anticipated (12).

In hydroxyl-containing solvents, nonhydrogenated ergot peptide alkaloids are readily epimerized at C-8 to an equilibrated mixture of the lysergic and iso-lysergic acid series, called the -ine/-inine forms (-ine = 8β , -inine = 8α) (12,18 and lit. quoted therein). The hydrolysis of the lysergic acid amide bond is of minor importance for the beginning degradation, but, of course, prevalent under more drastic hydrolysis conditions.

Under the same conditions, but at elevated temperatures, the C-2'-center is very likely to be inverted (aci-inversion) yielding a more acidic isomeric compound.

The light-induced addition of water to the 9,10-double bond of bromocriptine yielding the so-called lumi-products, is of high probability (12,18). They have, however, not yet been isolated or characterized. The corresponding 10 α -methoxy-lumi-derivative could be prepared by the photo-catalyzed addition of methanol (19) under slightly acidic conditions.

4.1 in Bulk

Although bromocriptine mesilate is sensitive to heat and light, it is stable for up to 3 years at ambient temperature (20) when stored in sealed polyethylene bags contained in twist-off amber glass bottles. In warm and tropical climate (30 °C/75 percent relative humidity) and under identical package conditions, it is stable for 1 year, however only for 3 months at 50 °C.

If not properly protected, bromocriptine adsorbs up to 6 percent by weight of water in tropical climate.

4.2 in Solution

Bromocriptine follows the behaviour sketched above and is thus rather labile in aqueous or aqueous/alcoholic solutions, particularly in the presence of acid, yielding mainly the equilibrated mixture with its 8-epimer (1) and to a smaller extent, its hydrolysis products 2-bromo-lysergamide and 2-bromo-lysergic acid and their 8 α -isomers, respectively.

4.3 in the Dosage Form

Bromocriptine mesilate is marketed as Parlodel® capsules (10 mg for the treatment of Parkinson's disease) and tablets (2.5 mg as lactation suppressor). Both forms have proved stable at least for 4 years when stored at ambient temperature in amber glass bottles (22).

5. Biopharmaceutical Aspects (23)

5.1. Pharmacokinetics

The disposition of bromocriptine has been studied in several animal species and man following single oral and intravenous administration of the drug labelled with either tritium or carbon-14.

The enteral absorption of bromocriptine from an aqueous solution amounts to 30 - 40 % as determined from the sum of the cumulative biliary and urinary excretion of radioactivity (parent drug + metabolites) in bile duct cannulated animals.

The blood levels following oral and intravenous doses are very low in all animal species. This, most likely, is due to the marked affinity of the drug for various tissues and the rapid hepatic extraction of the absorbed fraction. The main route of excretion is the bile. Less than 5 % of the dose are recovered in the urine of intact animals after oral or intravenous administration.

In man, the extent of enteral absorption is estimated to be at least of the same order of magnitude than determined for animals. Absorption is rapid with an approximate rate constant of 1.4 h^{-1} ($t^{1/2} = 30 \text{ min}$).

Peak plasma levels are reached about 1.5 h after oral ingestion, the maximum concentrations being in the order of 2 - 3 ng equivalents/ml (parent drug + metabolites) for an oral 1 mg dose. The elimination from the plasma is biphasic and proceeds with mean half-lives of 6 h (α -phase) and 50 h (β -phase). Similar elimination half-lives are obtained from the urinary excretion. The cumulative renal excretion is practically the same after oral and intravenous administration and amounts to 6 - 7 % of the radioactivity dosed. The main portion of the dose, either oral or intravenous, is eliminated by the biliary route into the faeces. The kinetics of bromocriptine has been demonstrated to be linear in the oral dose range from 2.5 to 7.5 mg.

Parent drug is bound to bovine and human plasma proteins to an extent of 89 - 96 % (in vitro concentration range 0.2 - 80 μ g/ml) and exerts a pronounced affinity to various tissues.

5.2 Metabolism

Bromocriptine is rapidly and completely metabolised in animals and man. The major components of the urinary metabolites have been identified as 2-bromo-lysergic acid and 2-bromo-isolysergic acid. Apart from the hydrolytic cleavage of the amine bond and the isomerization at position 8 of the lysergic acid moiety, a third principal biotransformation pathway consists in the oxidative attack of the molecule at the proline fragment of the peptide part, predominantly at position 8', giving rise to the formation of a number of hydroxylated and further oxidized derivatives of bromocriptine, and in addition of conjugated derivatives thereof.

6. Toxicology

The acute toxicity of bromocriptine mesilate has been determined in the mouse as 230 and 2620 mg/kg i.v. and p.o., respectively. In the rabbit, the corresponding values were 12 and >1000 mg/kg (2). Thus, bromocriptine proved less toxic than the nonhydrogenated ergot alkaloids by one order of magnitude, resembling the behaviour of the dihydrogenated derivatives.

Chronic toxicity studies were carried out with rats, dogs, and rhesus monkeys (2). In nearly all cases, the principal effect produced was ischemia of some part of the body. The well-known emetic effect of ergot alkaloids in dogs was particularly pronounced with bromocriptine, even with oral doses of as low as 0.1 mg/kg.

7. Analytical Methods

7.1 Application of General Tests

Compared with the unsubstituted ergot alkaloids (1,18) the introduction of bromine into the 2-position of the indole nucleus diminishes the reactivity of the molecule in respect to general alkaloid tests. Nevertheless, they still remain applicable.

1. Keller's test. Bromocriptine mesilate is dissolved in glacial acetic acid to which have been added traces of ferric chloride. After carefully layering with concentrated sulfuric acid, a green colour is produced at the interface. Usually an intense blue-violet colour occurs with 2-H-substituted compounds.

2. Van Urk's reaction. (24) Bromocriptine mesilate is dissolved in methanol. After addition of van Urk's reagent and vigorous shaking a blue colour develops slowly, which is substantially weaker than with 2-H-ergot alkaloids.

3. Meyer's test. Bromocriptine mesilate is dissolved in methanol/water 3:10. After addition of one drop of diluted hydrochloric acid and one drop of Meyer's reagent (mercuric potassium iodide) and shaking, a white precipitate is produced.

7.2 Titration

Bromocriptine mesilate may be assayed in glacial acetic acid/acetic anhydride 1:7 by titration with 0.1 N perchloric acid. The endpoint may be determined potentiometrically using a glass/calomel electrode system.

The methanesulphonic acid content of bromocriptine mesilate is usually determined by titration with 0.1 N methanolic potassium hydroxide. The endpoint may be detected potentiometrically using a glass/calomel electrode system.

Residual N-bromosuccinimide from the manufacturing process may be identified and/or quantified by making use of its oxidation potential by titration of liberated iodine after addition of potassium iodide in acetic acid (25).

7.3 Spectroscopic Methods

7.31 Infrared

Infrared spectroscopy is utilized for identification purposes during the analysis of the drug substance. (see 2.21)

7.32 Ultraviolet

Spectrophotometric analysis of bromocriptine mesilate is carried out directly using the uv maximum at about 305 nm in methanolic methanesulphonic acid. However, the method is not very specific.

It was preferred to first separate the impurities from bromocriptine by thin layer chromatography and then to isolate the substance by elution from the silica gel of the plate with methanol. The intact active ingredient is measured in 0.01 M methanolic methanesulphonic acid (26).

7.33 Colorimetry

In moderately acidic solutions bromocriptine mesilate readily forms ion pairs with anionic dyes such as picric acid, bromothymol blue, methyl orange, which are extractable with an organic solvent. A procedure has been developed both for direct assay and for assay following chromatographic separation from the impurities. Therein bromocriptine mesilate is allowed to react with bromothymol blue at pH 2.5. The resulting ion pair is then extracted with benzene and its concentration determined at 410 nm (25).

7.34 Proton Magnetic Resonance

PMR spectroscopy may be used for identification of the drug substance. (see 2.24)

7.4 Chromatography

7.41 Paper

Paper chromatography was applied formerly to the determination of bromocriptine.

conditions: system 1: stationary phase: 25 % formamide
mobile phase : carbon tetrachloride/
diethyl ether 1:1

system 2: stationary phase: 10 % dimethylphthalate
 mobile phase : dimethylformamide/
 0.5 N hydrochloric
 acid 15:85

The drug substance is visualized by reaction with iodine vapour.

Rf values of bromocriptine and precursor

	system 1	system 2
Bromocriptine	0.88	0.09
α -ergocryptine (precursor)	0.70	0.31

7.42 Thin Layer

The relative instability of bromocriptine makes it very difficult to avoid artifact formation in test solutions or during spotting on the plate. Therefore, bromocriptine mesilate, usually dissolved in chloroform/methanol 1:1, has to be spotted on the plate very rapidly and with the exclusion of light. The separation being terminated, the mobile phase is removed by means of high vacuum for 30 minutes.

A great number of tlc systems have been investigated for the separation of by-products, degradation products, metabolites, and excipients. Also a variety of spraying reagents have been tested (see table). The most advantageous one was Dragendorff's reagent with consecutive spraying by 30 % hydrogen peroxide.

methods: stationary phase: silica gel 60 F254, (27)
 Merck tlc plates, no.5715

mobile phase 1 : dichloromethane/dioxane/96 percent
 ethanol/conc.ammonia 180:15:5:0.1
 (v/v/v/v)

mobile phase 2 : chloroform/methanol/formic acid
 78:20:2 (v/v/v)

mobile phase 3 : chloroform/96 percent ethanol/conc.
 ammonia 192:8:0.35 (v/v/v)

In the procedure with mobile phase 1 and 2, the visualization is accomplished by screening under uv light (254 and 366 nm) and in addition by spraying with Dragendorff's reagent, modified by Munier and Deboeuf, followed by 30 percent hydrogen peroxide.

compound	R _{st} values	
	mobile phase 1	mobile phase 2
bromocriptine	1.0 (Rf 0.5)	1.0 (Rf 0.7)
2-bromo- α -ergocryptinine	1.5	1.1
α -ergocryptinine		
(isomer of precursor)	1.4	1.0
α -ergocryptine (precursor)	0.6	0.5
2-bromo-lysergic acid	0.0	0.25
2-bromo-isolysergic acid	0.0	0.35
2-bromo-lysergamide	0.5	0.15
2-bromo-isolysergamide	0.5	0.35

Mobile phase 3 may be used for the detection and semi-quantitative determination of residual N-bromosuccinimide. Thereby, the plate is sprayed with water and then placed in a chlorine atmosphere for 10 minutes. Excess chlorine is removed by placing the plate in a stream of warm air for another 10 minutes. After spraying with o-toluidine reagent, the evaluation is made against a dilution of N-bromosuccinimide. The detection limit is 0.1 μ g, the R_{st} is 0.3 with respect to bromocriptine Rf value.

A high performance tlc system has been developed for the in-process-control (28) using Merck silica gel HPTLC plates, no. 5628, as the stationary phase and tetrahydrofuran/chloroform/n-heptane/methanol/conc. ammonia 20:20:57:7:1 per volume as the mobile phase. The chromatography (6 cm ascending) is carried out without preceding chamber saturation. The compounds separated are visualized by uv at 254 and 366 nm, respectively, and by iodine vapour. Rf of bromocriptine is 0.27. The potential by-products yield spots at R_{st} 1.5 (2-bromo- α -ergocryptinine), and 0.55 (α -ergocryptine). 2-Bromo-lysergic acid remains at the starting point.

Beside the modified Dragendorff's reagent/hydrogen peroxide already mentioned, a variety of spraying reagents has been used for the visualization of bromocriptine. They are compiled in the following list.

BROMOCRIPTINE METHANESULPHONATE

spraying reagents for bromocriptine		
reagent	colour	detection limit on silica gel Merck no. 5715
<hr/>		
Dragendorff (Munier, Deboeuf)		
+ 30 percent H_2O_2	brown	0.2 μg
0.05 percent aqueous permanganate	yellow	1 μg
van Urk's reagent	grey	10 μg
cinnamic aldehyde	yellow	1 - 2 μg
formaldehyde/hydrochloric acid	grey	2 μg
formaldehyde/sulphuric acid	violet	0.1 μg
chlorine/o-toluidine	violet	0.1 μg
folin/ammonia	grey	1 μg
ninhydrin/uv det. 360	red	0.1 μg
iodine/potassium iodide	brown	2 μg
Ehrlich's reagent	orange	1 μg

The identity of methanesulphonic acid may be determined by tlc on cellulose plates, Merck no. 5728, with ethanol/water/conc. ammonia 80:16:4 (v/v/v) as a mobile phase. Detection is achieved by spraying with acid-base indicators, e.g. bromocresol green or similar species. Rf of methanesulphonic acid is 0.5 (that of bromocriptine base = 0.9).

7.43 Gas Chromatography

GC cannot be applied to the analysis of bromocriptine mesilate due to its low volatility and its thermal instability. A procedure according to 29 or 30, which claims excellent identification and quantitation on the basis of well-defined peptide section pyrolysis products, has not yet been attempted. However, GC is very useful determining the residual recrystallization solvent butanone-2. The conditions are the following:

Column: Porapak Q 80/100 mesh, in 1.8 m x 2mm glass
Temperatures: injector 240 °C, column 130 °C
flame ionization detector, 240 °C.

7.44 High Performance Liquid

A series of HPLC systems have been utilized both for assay and purity of bromocriptine mesilate. Satisfactory procedures have been accomplished both on straight phase (silica gel) and on reversed phase (octadecylsilanised silica gel) columns.

HPLC-conditions

straight_phase:

stationary phase: silica gel, 5 μ m, in stainless steel
25 cm x 3 mm i.d.
mobile phase: water-saturated dichloromethane/methanol
100:2 (v/v)
uv detection at 254 nm.

Fig. 11 shows a chromatogram of bromocriptine mesilate spiked with dodecylbenzene, potential by-products and the precursor. The flow was 1.7 ml/minute (31).

Key: 1 = dodecylbenzene, 2 = bromocriptinine, 3 = α -ergocryptinine, 4 = bromocriptine, 5 = α -ergocryptine (precursor).

reversed_phase, system_I:

stationary phase: Merck RP-18, 10 μ m in stainless steel
25 cm x 4.6 mm i.d.
mobile phase: gradient: 35 to 60 percent B within 60 min.
A = water, 0.2 percent triethylamine added
B = acetonitrile, 0.2 percent triethylamine added.
uv detection at 280 nm.

This system was found optimal for the purity test.

Fig. 12 shows a chromatogram of the drug substance spiked with potential by-products and the precursor. The flow was set at 4.0 ml/minute.

Key: I = 2-bromo- α -ergocryptinine, II = α -ergocryptinine, III = α -ergocryptine (precursor), IV = bromocriptine, V = 2-bromo-lysergamide, VI = 2-bromo-lysergic acid, VII = 2-bromo-isolysergamide.

reversed_phase, system_II (26):

stationary phase: Merck RP-18, 10 μ m in stainless steel,
25 cm x 4.6 mm
mobile phase: 0.01 M ammonium carbonate or 0.05 M
ammonium hydrogen carbonate solution/
acetonitrile 35:65 isocratic,
flow 1.5 ml/min.

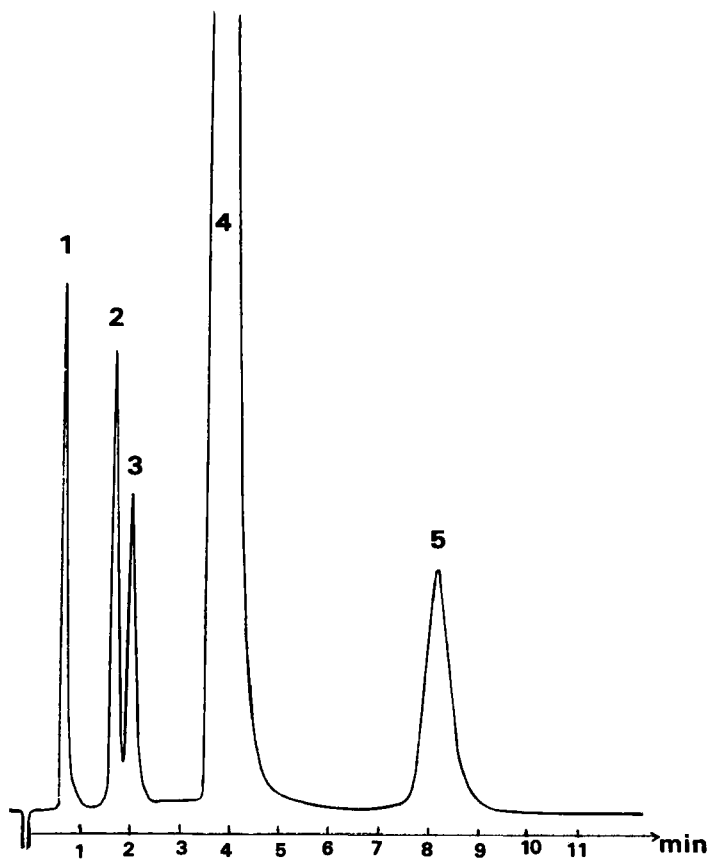


Figure 11. High Performance Liquid Chromatogram of Bromocriptine Mesilate, spiked with Dodecylbenzene, Precursor and potential By-products. Adsorption Mode, isocratic, Uv-detection at 254 nm

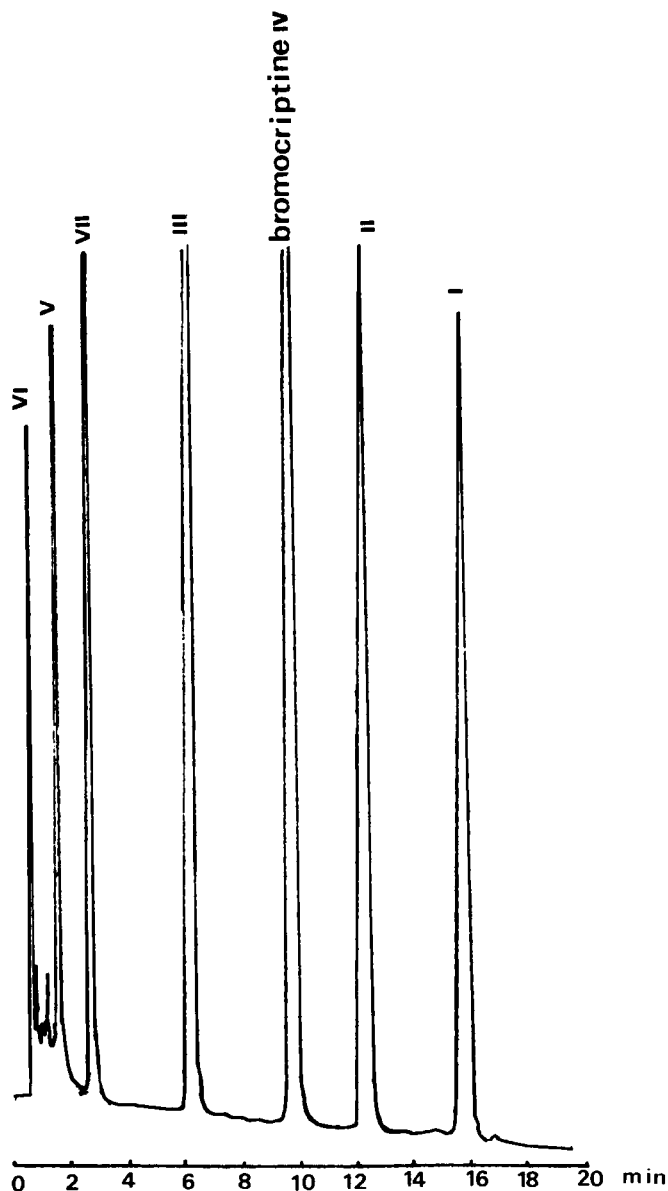


Figure 12. High Performance Liquid Chromatogram of Bromocriptine Mesilate, spiked with Precursor and potential By-products. Reversed-phase Mode, Solvent Gradient, Uv-detection at 280 nm.

7.5 Differential Scanning Calorimetry

Differential scanning calorimetry cannot be applied to quantify the purity of the drug substance for the reasons mentioned in 2.31 and 2.34. However, it may be valuable in qualitative comparisons from sample to sample or batch to batch on the basis of their corresponding differential scanning calorimetry patterns.

7.6 Phase Solubility Analysis

For phase solubility analysis, acetonitrile appears to be the most suitable solvent. A typical plot is given in figure 13 along with the experimental conditions.

7.7 Analysis of the Dosage Form

The identification of bromocriptine mesilate in the dosage form can be carried out by thin layer chromatography using Merck plates with dichloromethane/methanol/formic acid 78:20:2 (v/v/v) and subsequent uv-visualization at 254 and 360 nm. Using this method, it is important to only air-dry the spot after application to the plate, since more vigorous evaporation of the solvent will give rise to artifacts (32).

Bromocriptine can also be identified as the base by ir spectroscopy after extraction from the dosage form with ethanol and removal of the solvent, both in solution and in a KBr pellet (33).

Bromocriptine mesilate in Parlodel® tablets may be assayed in a non-specific way by direct uv-spectrophotometry following extraction with methanol (32).

Fluorimetry in 0.1 N hydrochloric acid has been applied during the measurement of dissolution rate of the dosage forms with excitation at 335 and emission at 425 nm, respectively (26).

A specific assay of bromocriptine mesilate in the dosage form may be carried out by tlc followed by uv-spectrophotometry (26) (The system can also serve for identification purposes). The drug substance is extracted with methanol in the absence of light, the chromatographic conditions are: Merck plates F 254, mobile phase: dichloromethane/dioxane/ethanol abs./conc. ammonia 180:15:5:0.1 per volume. The spot corresponding to bromocriptine is extracted with methanol, and the concentration is determined at about 300 nm by spectrophotometry.

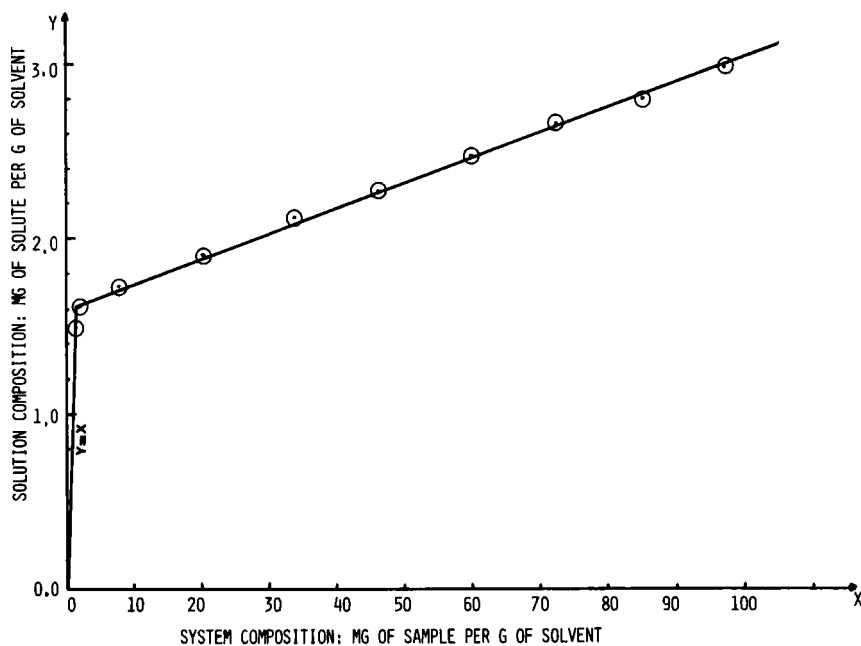


Figure 13. Phase Solubility Analysis Plot of Bromocriptine Mesilate, dried in High Vacuum for 15 Hours. Solvent: dry Acetonitrile, Vibration for 24 hrs. in the Absence of Light. Slope 1.41 ± 0.05 %.

A further specific assay is the HPLC-determination of bromocriptine mesilate following extraction with methanol from the dosage form using RP-18 as the stationary and acetonitrile/0.01 M ammonium carbonate solution 65:35 as the mobile phase. Uv-detection wavelength is set at 300 nm (26).

For the detection and estimation of degradation products in dosage forms, a solvent gradient, containing the components mentioned above, is utilized with advantage.

7.8 Determination in Body Fluids and Tissues (23)

Due to its potent efficacy in the treatment of hyperprolactinemia and acromegaly, bromocriptine is administered in low doses leading to minute concentrations in body fluids and tissues. Therefore, none but the most sensitive analytical methods can be used to measure its concentration in biological specimens. The only method so far applicable for pharmacokinetic studies with bromocriptine is the use of the radioactively labelled drug, measurement of total radioactivity and its fractionation by chromatographic separation techniques for the assay of parent drug and major metabolites. Recently, a radioimmunoassay kit for the analysis of picogram quantities of unchanged bromocriptine in body fluids has become available. Gas chromatography, mass fragmentography and liquid chromatography also appear to be suitable for determining bromocriptine in plasma from patients with Parkinson's disease which are on treatment at high dose levels. These currently developed procedures permit quantitative determinations down to concentrations of 0.5 (GC), 1.0 (MF), and 10 ng/ml (LC), respectively (34).

The pattern of metabolites in bile (animals only) and in urine have been investigated using column chromatography (Amberlite XAD 2 and Sephadex DEAE), tlc and reversed phase HPLC in combination with radioactivity monitoring. The principal metabolites have been isolated from the bile of rats treated with high doses of bromocriptine. The structure of the isolated metabolites was elucidated by means of spectroscopic techniques.

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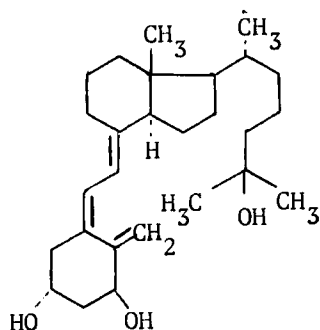
CALCITROL

Eileen Debesis

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1. Description1.1 Name, Formula, Molecular Weight

Calcitriol is 1 α , 25-dihydroxycholecalciferol.



MW = 416.65

$C_{27}H_{44}O_3$

1.2 Appearance, Color, Odor

Calcitriol is an odorless white crystalline powder.

2. Physical Properties2.1 Infrared Spectrum

The infrared spectrum of calcitriol is shown in Figure 1 (1). The spectrum was recorded on a Perkin-Elmer Model 283 Grating Infrared Spectrophotometer and was measured in a KBr pellet which contained 1 mg of calcitriol in 300 mg of KBr.

The following absorptions have been assigned for

Figure 1:

- a. OH stretching (bonded): 3391 cm^{-1}
- b. Aliphatic CH stretching: $2943, 2872\text{ cm}^{-1}$
- c. CH deformation: $1468, 1378\text{ cm}^{-1}$
- d. C-O stretching: 1056 cm^{-1}

2.2 Nuclear Magnetic Resonance Spectrum (NMR)

The NMR spectrum of calcitriol, recorded on a Varian XL-100/Nicolet TT-100 pulsed Fourier Transform NMR spectrometer, with internal deuterium lock, is shown in Figure 2 (2). The spectrum was recorded using a solution of 0.84 mg of sample dissolved in 50 microliters of CD_3OD (100%D) containing 1% v/v tetramethylsilane in a 1.7 mm capillary tube. The spectral assignments are given in Table I.

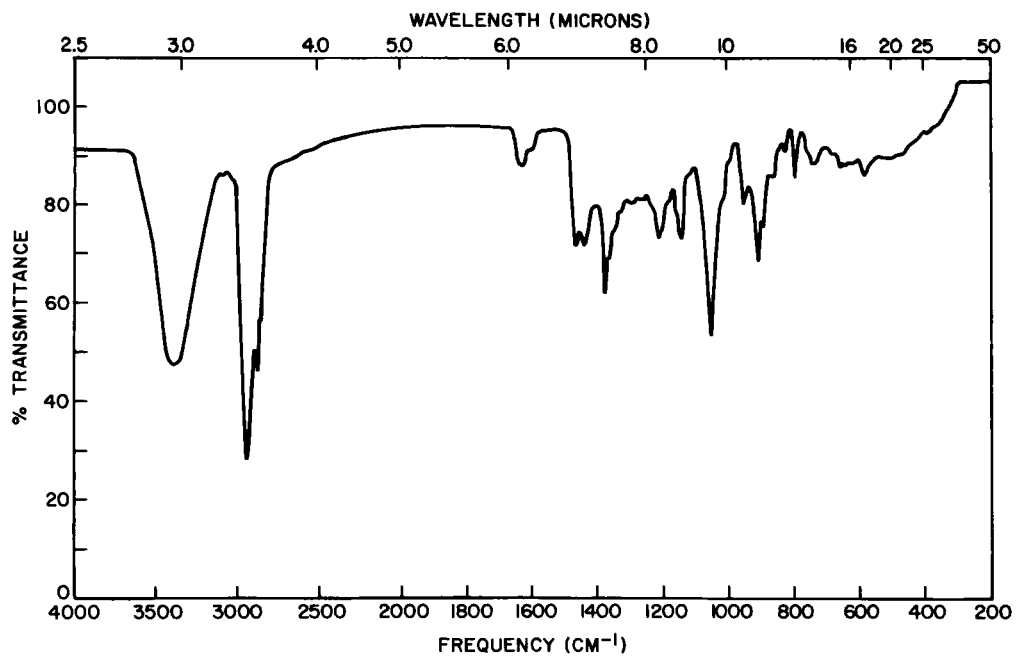


Figure 1
Infrared Spectrum of Calcitriol

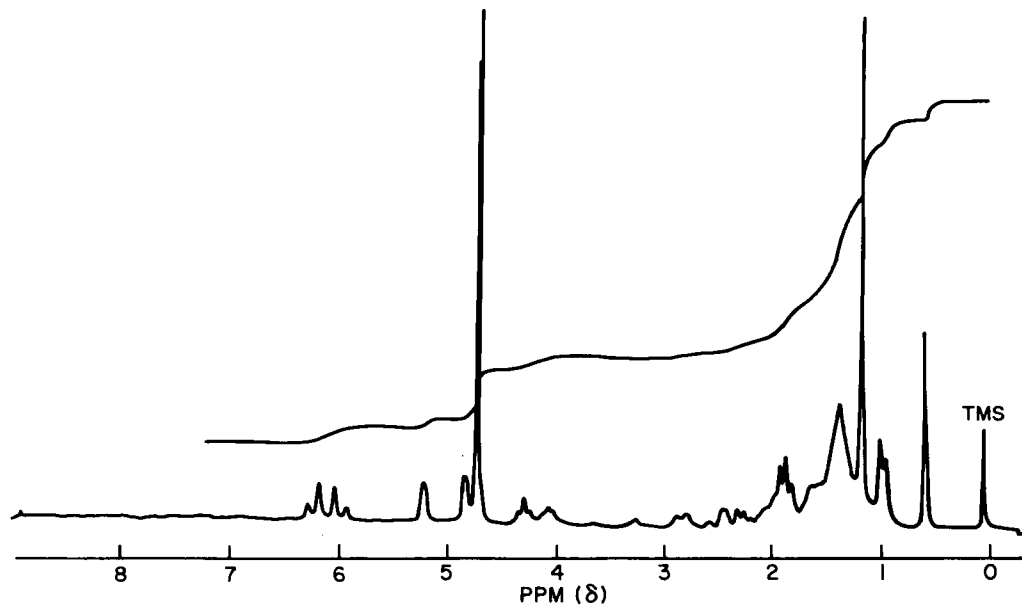


Figure 2
NMR Spectrum of Calcitriol

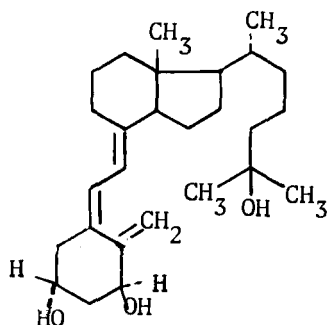


Table I

NMR Spectral Assignments for Calcitriol

<u>Proton</u>	<u>Chemical Shift (σ)</u>	<u>Multiplicity</u>
$-\text{CH}_3(\text{C}_{18})$	0.57	Singlet
$-\text{CH}_3(\text{C}_{21})$	0.95	Doublet
$-\text{CH}_3(\text{C}_{26,27})$	~ 1.16	Singlet
$\text{>CH}_2, \text{>CH}$	$\sim 1.38-3$	Complex
$\text{HO}-\text{CH}$	4.08	Complex
$\text{HO}-\text{CH}$	4.32	Triplet
$=\text{CH}_2$	4.90, 5.28	Complex
$\text{H}-\text{C}=\text{C}-\text{H}$	6.07, 6.32	AB Quartet $J_{AB} = 11 \text{ Hz}$
HDO + 3 x OH (exchangeable protons)	4.78	-

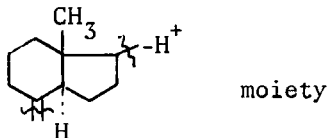
2.3 Ultraviolet Spectrum

The ultraviolet spectrum of calcitriol (1 mg of calcitriol/100 ml of absolute ethanol) in the region of 220 to 400 nm exhibits one maximum at 264 nm ($\epsilon = 1.9 \times 10^4$) and one minimum at 226 nm. The spectrum is shown in Figure 3 (3).

2.4 Mass Spectrum

The low resolution mass spectrum of calcitriol is shown in Figure 4 (4). The spectrum was obtained using a Varian MAT CH5 spectrometer, which was interfaced with a Varian data system 620 I. The data system accepts the output of the spectrometer, calculates the masses, compares the intensities to the base peak, and plots this information as a series of lines whose heights are proportional to the intensities.

The molecular ion was measured at $m/e = 416$. Other characteristic masses were observed at $m/e = 398$, 380 and 362, corresponding to the loss of one, two and three molecules of water, respectively, from the molecular ion; $m/e = 383$, corresponding to the loss of water and CH_3 from the parent peak; and $m/e = 365$, corresponding to the loss of two water molecules and one CH_3 from the molecular ion. The base peak is observed at $m/e = 134$, and corresponds to the



2.5 Melting Range

Calcitriol melts at 111-115°C (3).

2.6 Differential Scanning Calorimetry

Melting was accompanied by decomposition (5).

2.7 Thermogravimetric Analysis

Calcitriol was subjected to thermogravimetric analysis on a Perkin-Elmer Model TGS-1 Thermogravimetric Analyzer. The sample exhibited two overlapping weight losses. The initial weight loss, of 0.4%, began at 55°C and ended at 105°C, and was due to surface moisture or solvent. The second weight loss, due to decomposition, amounted to 2.5% at 205°C, 7.5% at 255°C, 33% at 305°C, and 93% at 355°C (5).

2.8 Hot Stage Microscopy

As observed on a Mettler Hot Stage FP 52 with FP5 controller, the sample appeared as birefringent needle-like crystals which melted from 115-117°C. The sample did not recrystallize from the melt (5).

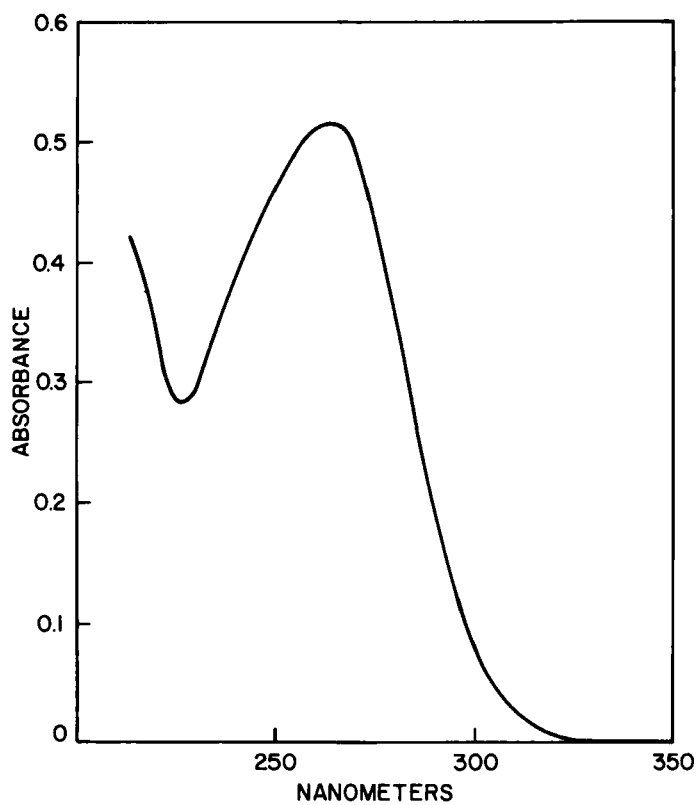


Figure 3
Ultraviolet Spectrum of Calcitriol

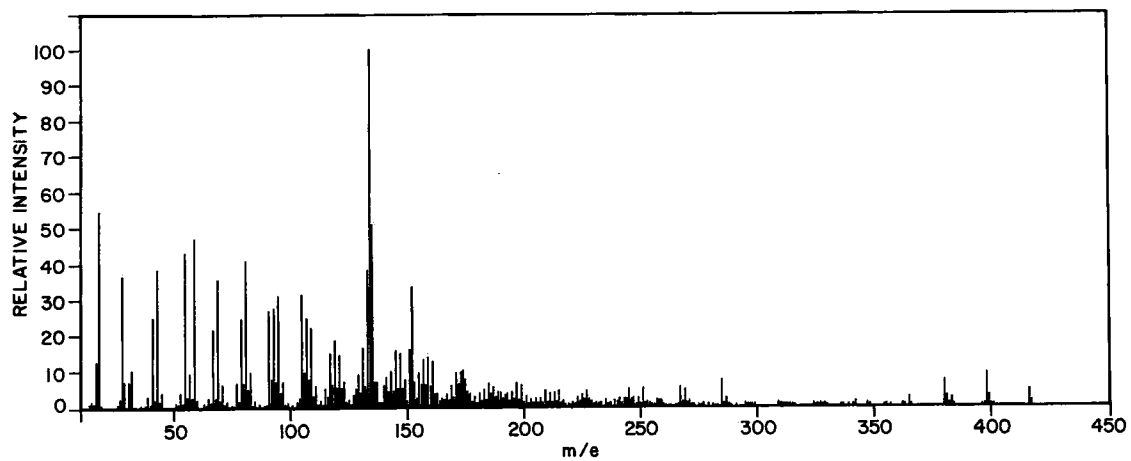


Figure 4
Mass Spectrum of Calcitriol

2.9 Solubility

Extensive solubility data is not readily obtainable due to the scarcity of calcitriol. The material is slightly soluble in methanol, ethanol, ethyl acetate and tetrahydrofuran (3).

2.10 X-Ray Powder Diffraction

The X-ray powder diffraction data for calcitriol are presented in Table III (3); instrumental conditions are given below. The diffraction pattern is shown in Figure 5.

Instrument and Operating Conditions

Instrument	Guinier-De Wolff Camera
Generator	GE XRD-6 50 KV, 12.5 mA
Detector	Film
Sample	As is
Densitometer*	Gelman DCD-16
Optics	Tungsten Lamp 575 nm Visible mode
Detector	Silicon phototransistor
Optical density	0.25
Beam exit slit	0.53 mm

*The diffraction pattern was recorded on film; the density of the individual lines in the pattern was determined by densitometry.

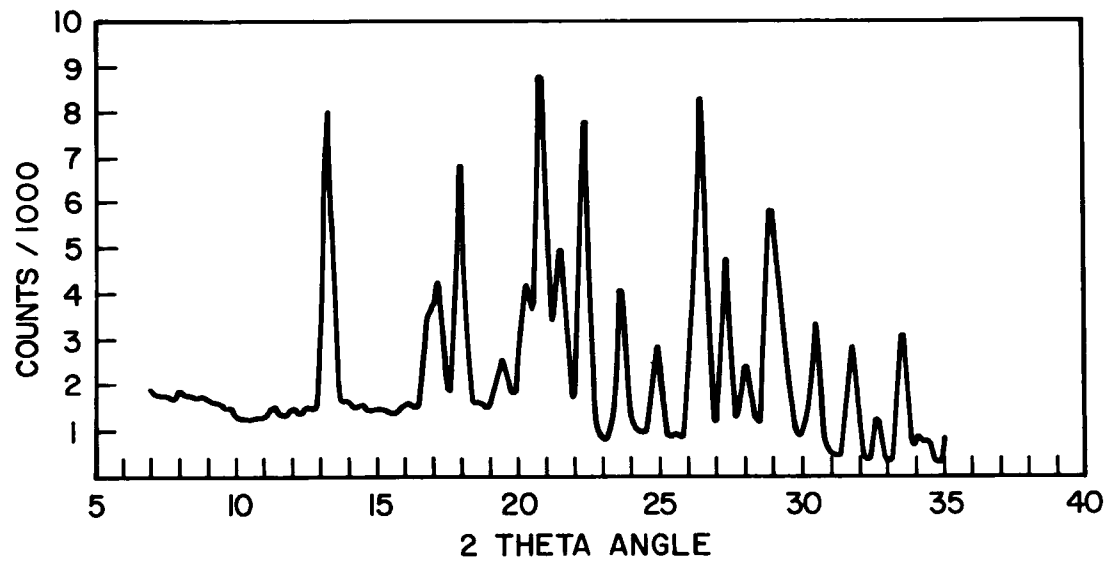


Figure 5
X-Ray Powder Diffraction Pattern of Calcitriol

TABLE III
Calcitriol Powder Diffraction Data

<u>2θ</u>	<u>d (Å) *</u>	<u>I/Io**</u>
26.52	4.993	100
13.33	9.863	90
22.41	5.892	88
20.89	6.316	82
18.01	7.314	70
28.97	4.578	66
27.33	4.847	49
23.70	5.577	44
33.55	3.968	36
30.54	4.348	34
31.77	4.183	33
17.19	7.662	33
24.95	5.302	26
21.54	6.129	26
28.04	4.727	15
32.66	4.072	12
19.46	6.776	12
20.35	6.480	12
16.07	8.192	2
11.37	11.559	2
8.03	16.343	2
34.18	3.896	2
12.07	10.889	1

*d = $\frac{n \lambda}{2 \sin \theta}$ (interplanar distance)

**I/Io = percent relative intensity (based on maximum intensity of 1.00)

2.11 Optical Rotation

The specific rotation of calcitriol in absolute methanol, measured at 589 nm and 25°C, was +48° (7).

3. Synthesis

Several procedures for the synthesis of calcitriol have been reported in the literature. Parren et al. (8) and Norman et al. (9) described the preparation of calcitriol from 25-hydroxyvitamin D₃. Uskokovic and others (10-18) have described lengthier syntheses utilizing more readily available starting materials. A typical reaction scheme, utilizing 1 α , 25-diacetoxy-7-dehydrocholesterol as the starting material, is shown in Figure 6.

4. Stability and Degradation

Calcitriol must be protected from air and light. The drug substance exhibits good stability when stored at -15°C to -25°C in an argon atmosphere. The material is stable at room temperature when dissolved in a vegetable oil derivative, containing antioxidants, such as is used in calcitriol soft gelatin capsules (19).

5. Drug Metabolic Products

Calcitriol may be absorbed directly into the intestine or bone, or may be hydroxylated to form 1 α , 24, 25-trihydroxycholecalciferol prior to intestinal absorption (20-24).

6. Methods of Analysis

6.1 Elemental Analysis

A typical elemental analysis of a sample of calcitriol is presented in Table IV (7).

TABLE IV

Elemental Analysis of Calcitriol

<u>Element</u>	<u>% Theory</u>	<u>% Found</u>
C	77.84	77.53
H	10.64	10.75
O	11.52	11.92
		(by difference)

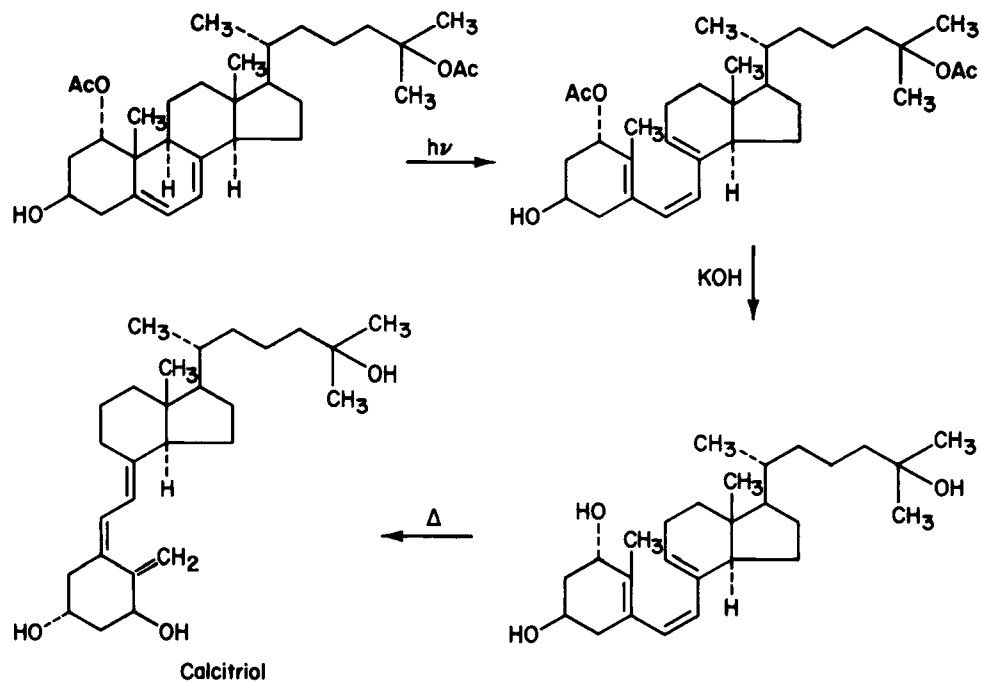


Figure 6
Synthesis of Calcitriol

6.2 Chromatographic Methods

6.21 Thin-Layer Chromatography (TLC)

The following TLC procedure is useful for determining the purity of calcitriol. It separates the pre-vitamin, 1 α , 25-dihydroxyprecholecalciferol. A silica gel GF plate is activated by heating for one hour at 105°C and is then cooled in a desiccator. A low actinic all glass chromatographic chamber is equilibrated with the developing solvent, and 0.8 mg of calcitriol is applied to the plate from ethyl acetate. The plate is developed in an ascending mode in ethyl acetate:spectroquality heptane:methanol (100:10:2) for 15 cm. After air drying, the plate is viewed under shortwave ultraviolet radiation, then sprayed with a 15% w/v solution of phosphomolybdic acid in ethanol, followed by heating at 105°C for 10 minutes to develop the colors. The approximate R_f values are summarized in Table V (3).

TABLE V

Summary of TLC Data

<u>Compound</u>	<u>Approximate R_f</u>
1 α , 25-Dihydroxyprecholecalciferol	0.4
Calcitriol	0.5

6.22 High Performance Liquid Chromatography

High performance liquid chromatography is used to determine the purity of calcitriol, and to separate it from related compounds. Using a 10 micron silica column of 25 cm length, and a mobile phase of spectroquality heptane:ethyl acetate:methanol (50:50:1) at a flow rate of 1.7 ml/minute, separation and quantitation are achieved. p-Dimethyl-aminobenzaldehyde may be used as an internal standard to compensate for variations in injection technique and instrumental conditions. With a 254 nm ultraviolet absorbance detector, 0.01 μ g of calcitriol may be detected (3).

This procedure, with a mobile phase of spectroquality heptane:ethyl acetate:methanol (70:25:5) is also useful for analyzing calcitriol in soft gelatin capsules. The capsule fill solution may be injected directly. The amount of calcitriol in the capsule is determined by comparison to a calcitriol reference standard, prepared in a medium similar to the capsule fill (3).

6.23 Gas Chromatography - Mass Spectrometry

Halket and Lisboa (25) examined several Vitamin D derivatives by capillary gas chromatography coupled with mass spectrometry. This technique offered the advantages of great sensitivity and separating power. Retention times and fragmentation patterns for ergocalciferol, cholecalciferol and calcitriol were reported.

6.3 Biological Methods

6.31 Radioimmunoassay

Several workers (26-32) have reported on the use of radioimmunoassay for measuring calcitriol at very low (pg) levels in serum.

6.32 Protein Binding Assays

Various protein binding techniques are reported (32-42) for the determination of calcitriol in plasma. Generally, a preliminary purification step is required to avoid interference from other plasma components.

6.33 Bioassays

Stern, et al. (43,44) reported a bioassay technique based on fetal rat bone absorption of calcitriol. Parkes and Reynolds (45) developed an in-vitro bioassay using duodenal tissue from chicken embryos.

6.4 Polarography

Calcitriol drug substance may be analyzed polarographically, using a glassy carbon working electrode. The limiting current of the observed oxidation wave ($E_{1/2} = 0.96$ v) is linear with concentration in the 0.01 to 0.03 mM region (46).

7. Acknowledgments

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CHLORTETRACYCLINE HYDROCHLORIDE

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¹Retired.

1. DESCRIPTION

1.1 Drug Properties

Chlortetracycline hydrochloride (CTC-HCl) is the hydrochloride salt of an antibiotic substance produced by the growth of Streptomyces aureofaciens (Fam. Streptomycetaceae). It was discovered in 1948 by Duggar (1).

CTC-HCl is a broad-spectrum antibiotic and anti-protozoan active against many Gram-positive bacteria, some Gram-negative bacteria, spirochetes, amebae, and certain large viruses. Staphylococci have become generally resistant to the drug, and drug-resistant strains may be found among other bacterial genera and species generally sensitive to it. Cross-resistance to other antibiotics of the tetracycline family is automatic (2).

Organisms may be considered susceptible if the Minimum Inhibitory Concentration (MIC) is not more than 4.0 µg/ml and intermediate if the MIC is 4.0-12.5 µg/ml (see Table 1). Tetracyclines are readily absorbed and are bound to plasma proteins in varying degrees. They are concentrated by the liver in the bile and excreted in the urine and feces at high concentrations and in a biologically active form. The mode of action against microorganisms involves the inhibition of phosphorylation processes in bacterial cells (3).

TABLE 1 (5)
Antimicrobial Spectrum of Chlortetracycline

Microorganism	Minimum Inhibitory Concentration (µg/ml)
<u>Micrococcus pyrogenes</u> var. <u>aureus</u> 209P	0.292
<u>Micrococcus pyrogenes</u> var. <u>aureus</u> 1248A	0.39
<u>Streptococcus pyrogenes</u>	0.29
<u>Streptococcus mitis</u>	0.147
<u>Streptococcus faecalis</u>	0.39
<u>Micrococcus flavus</u>	0.292
<u>Diplococcus pneumoniae</u>	0.098
<u>Sarcina lutea</u>	0.147
<u>Bacillus subtilis</u>	0.195
<u>Escherichia coli</u>	1.45
<u>Haemophilus influenzae</u>	0.312
<u>Klebsiella pneumoniae</u>	0.29
<u>Neisseria catarrhalis</u>	0.098
<u>Aerobacter aerogenes</u>	1.17
<u>Proteus vulgaris</u>	4.6
<u>Pseudomonas aeruginosa</u>	25
<u>Salmonella schottmuelleri</u>	3.12
<u>Salmonella typhi</u>	1.17
<u>Shigella dysenteriae</u>	3.12
<u>Brucella bronchiseptica</u>	0.292
<u>Mycobacterium tuberculosis</u>	0.147
<u>Mycobacterium friedmanii</u>	0.122
<u>Mycobacterium smegmatis</u>	0.073
<u>Mycobacterium</u> sp.	0.58
<u>Candida albicans</u>	100
<u>Clostridium butyricum</u>	0.078
<u>Pasteurella multocida</u>	0.049
<u>Vibrio parcolans</u>	0.098

1.2 Physical Description and Optical Crystallography

CTC-HCl is a yellow, odorless powder composed mainly of crystals in the shape of small hexagons, and has the following optical crystallographic characteristics (4):

α : 1.635; optic sign negative; $2\nu = 59^\circ$ (calc.);

β : 1.706; orthorhombic; extinction parallel; and

γ : 1.730; symmetrical

It is stable in air, but is slowly affected by light (6).

1.3 Chemical Properties

CTC-HCl is the HCl salt of amphoteric CTC; it is multifunctional with two chromophores. It is a para-chlorophenol with an α,β -unsaturated ketone in conjugation. The second chromophore involves another α,β -unsaturated ketone that is in conjugation with an anomalously behaving amide (7). The tertiary amine is responsible for the basic character and the phenolic group is acidic. CTC is fluorescent and can be assayed polarographically (8).

A particularly intriguing aspect of the chemistry of the compounds of the tetracycline family is their ability to form metallic complexes. CTC shares in this intensively studied capability, which is very likely related to the therapeutic activity (9). This property is also used in the purification (10) and analysis (11) of CTC.

1.4 Structure

$C_{22}H_{23}ClN_2O_8.HCl$ Empirical Formula

515.35 Molecular Weight

7-Chloro-4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide monohydrochloride (CAS-64-72-2) (6,12-14) (Figure 1).

1.5 The FDA Chlortetracycline Standard

The current official FDA Chlortetracycline Working Standard is chlortetracycline hydrochloride, Lot #501-632B-95-1 (9/29/53), obtained from Lederle, which markets the antibiotic under the proprietary name Aureomycin. The current working standard has an assigned potency of 1000 $\mu\text{g}/\text{mg}$ (the term μg applied to chlortetracycline means the chlortetracycline activity (potency) contained in 1 μg of the FDA Chlortetracycline Master Standard (Lot #990-107-141-1), which is also chlortetracycline hydrochloride).

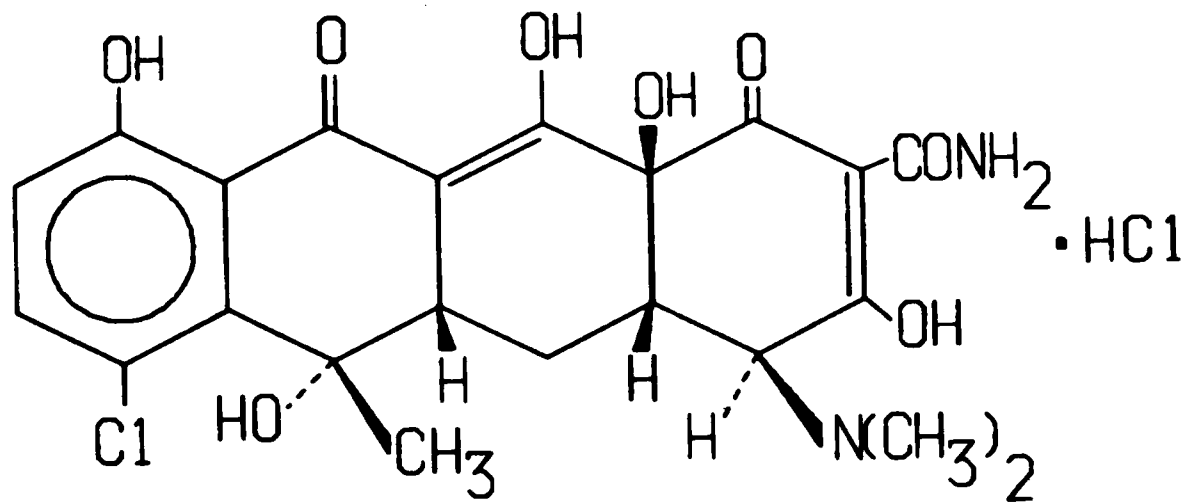


Figure 1. Structural formula of CTC-HCl.

The Working Standard is stored in lots of 250 mg at -20°C , protected from light and moisture, at the National Center for Antibiotics Analysis, Washington, DC.

2. PHYSICAL PROPERTIES

2.1 Thermal Properties

Differential Thermal Analysis (DTA) and Thermal Gravimetric Analysis (TGA). CTC-HCl is stable until it begins to decompose exothermically at approximately 230°C (Figures 2 and 3) (15). The compound does not lose any mass until the final decomposition takes place. No polymorphs have been seen in the samples examined.

2.2 X-Ray Powder Diffraction

The X-ray diffraction pattern of the FDA Working Standard has been determined. It demonstrates crystalline structure; the data are listed in Table 2 (16).

TABLE 2
X-Ray Diffraction Data

d(Å)	I/I ₁	d(Å)	I/I ₁	d(Å)	I/I ₁
		3.34	17		
9.10	11	3.27	37	2.060B	13
8.48	12	3.23	16	^D (1.985	11
^D (7.75 ^a	56	^D (3.17	73	(1.962	13
^D (7.43	44	^D (3.12	74	1.897	12
6.65	13	3.095	38	1.880	10
6.38	43	2.982	8	1.868	13
^D (5.72	81	2.948	9	1.856	13
^D (5.61	42	2.910	47	1.827	8
5.28	61	^D (2.878	25	1.805	9
5.16	8	^D (2.854	31	1.784	6
^D (4.96	8	2.788	35	^D (1.748	8
^D (4.88	15	2.710	36	(1.737	11
4.68	3	^D (2.653	14	1.705	9
4.54	40	^D (2.632	10	1.682	6
4.42	100	2.560	30	1.638	5
4.29	70	2.482	22	1.631	4
4.24	25	2.440	13	1.608	5
^D (4.15	52	2.421	27	1.595	4
^D (4.09	78	2.390	12	1.583	4
3.88	37	2.343	24		
3.80	31	2.290	7		
3.71	59	2.235	14		
3.64	33	2.210	37		
3.57	16	2.170	13		
3.52	25	2.142	23		
3.37	23	2.093	21		

^aD = Doublet.

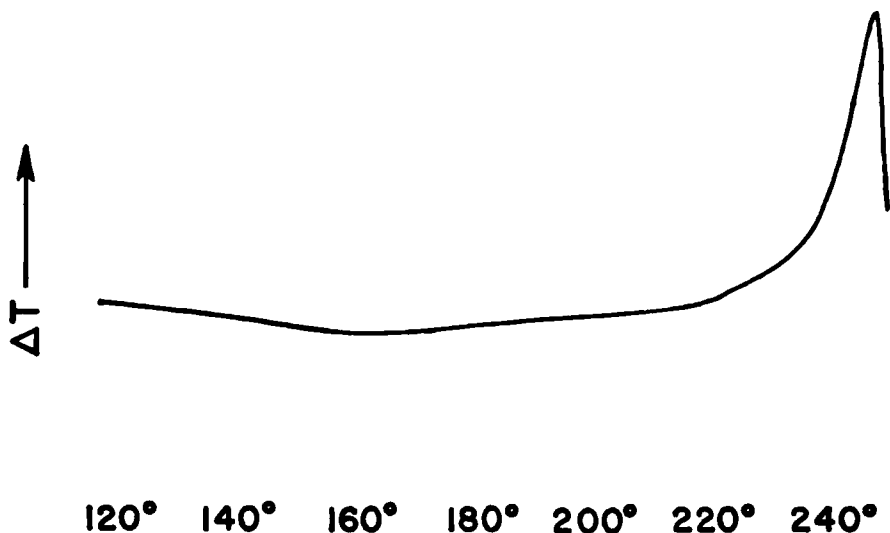


Figure 2. Differential thermogram of CTC-HCl.

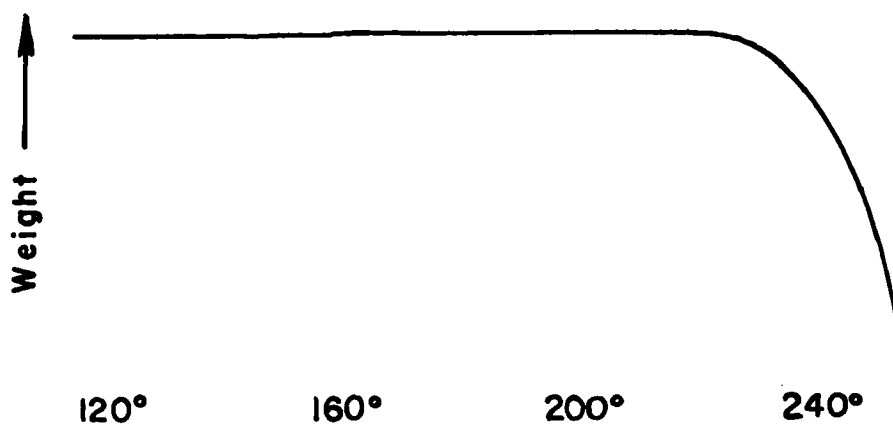


Figure 3. Thermal gravimetric analysis curve of CTC-HCl.

2.3 Solubility

The solubility of antibiotics, including CTC-HCl, was reported by Andrew and Weiss (17). CTC-HCl is an amphoteric substance and consequently it is soluble in aqueous acid and base. However, it can rapidly degrade in these solvents. Its solubility in water is about 8 mg/ml and in methanol about 17 mg/ml. In higher molecular weight alcohols, the solubility of CTC-HCl is considerably less than in methanol. For practical purposes, it is insoluble in many common solvents such as the aliphatic hydrocarbons, benzene, ether, and chloroform. It is readily soluble in pyridine and to the extent of about 5 mg/ml in formamide. Pyridine is an undesirable solvent because of its basicity, and formamide is not desirable because of the difficulty in obtaining and maintaining it as a stable solvent.

2.4 Acid-Base Properties

CTC exhibits three acidic dissociation constants when titrated in aqueous solutions (18). Stephens et al. (19) identified the three acidic groups (Figure 4), and reported thermodynamic pKa values of 3.30, 7.44, and 9.27. Leeson et al. (20) assigned pKa values to the following acidic groups:

<u>pKa</u>	<u>Assignment</u>
3.30	Tricarbonylmethane System (A)
7.44	Phenolic Diketone System (B)
9.27	Dimethylamino System (C)

Kalnins and Belen'skii (21) verified the assignments by infrared (IR) spectroscopy.

The pH of a 10 mg/ml aqueous solution, as described in the Code of Federal Regulations monograph for CTC-HCl (22) should lie between 2.3 and 3.3.

2.5 Polymorphism

In recent years, with growing concern about the relative bioavailabilities of different samples of the same drug substance, polymorphism has become of prime interest. Miyazaki and co-workers (23) have reported the existence of two crystalline forms of CTC-HCl. The X-ray powder diffraction patterns, IR spectra, dissolution behaviors, and hygroscopicities that they reported were distinctly different and there were discrepancies in the bioavailabilities. In later work on two forms of CTC base prepared by crystallizing from water or methanol, the X-ray patterns and

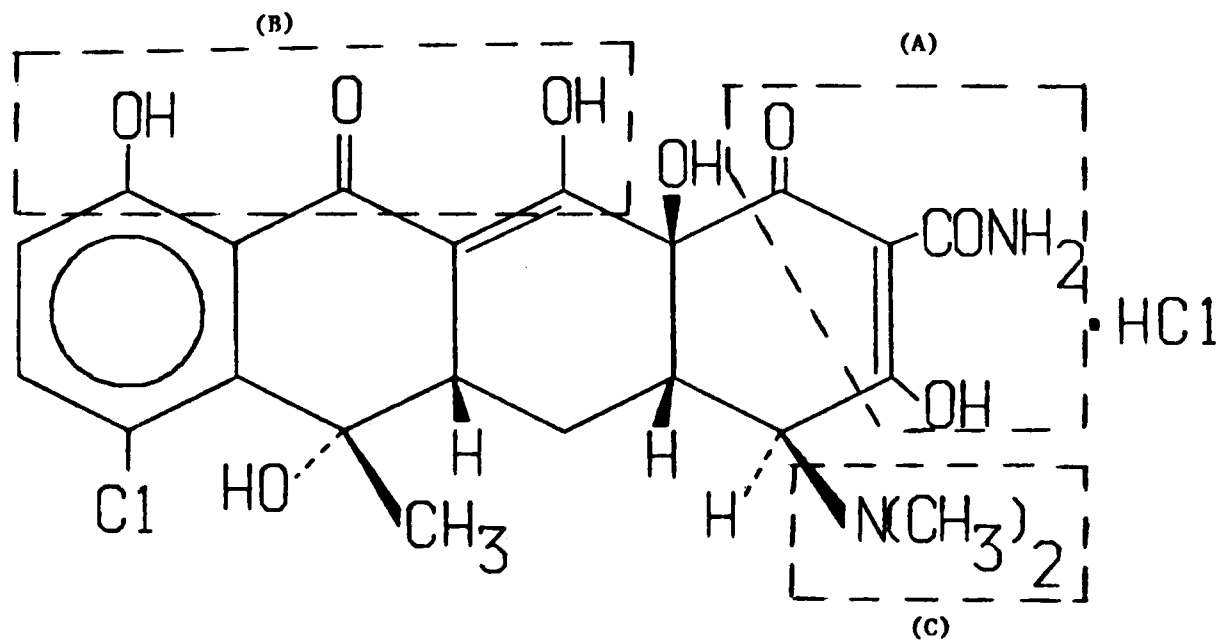


Figure 4. Acid-base properties of CTC.

water contents were different, but other physical properties were indistinguishable. There appeared to be no difference in bioavailability (24). No polymorphs were found in the FDA CTC standard.

3. SPECTRAL PROPERTIES (OPTICAL)

3.1 Ultraviolet

<u>Medium</u>	<u>Maxima (nm)</u>
0.1N HCl	368, 340sh, 322sh, 265, 229
0.1N NaOH	345, 283, 253, 222
Methanol	372, 342sh, 322sh, 262sh, 253, 233

See Figure 5 (25).

3.2 Infrared

The IR absorption spectra of CTC-HCl are shown in Figures 6 and 7. Spectra were run on a Perkin-Elmer Model 467 grating spectrophotometer as a KCl pellet (1 mg/200 mg KCl), and as a Nujol mull (20 mg/3 drops). The spectra are essentially identical. The CTC-HCl spectrum may also be found in a compilation of IR spectra of Drug Reference Standards by Hayden et al. (26). Major absorption frequencies have been compiled (27) and assignments have been made on many of the bands(28-31). Some of the major frequencies and band assignments are:

<u>cm⁻¹</u>	<u>λ</u>	
3360	2.98	νNH of NH ₂ (asym.)
3315	3.02	νNH of NH ₂ (sym.)
3200-3450	2.90-3.12	νOH (alcoholic, masks NH)
2800-2400	3.57-4.17	tertiary amine halide salt, group of broad bands
1675	5.97	νC=O
1582	6.32	δNH ₂
1450	6.90	δCH (bending of CH ₃)
1368	7.31	νCN
1042	9.60	OH (deformation, alcohols)

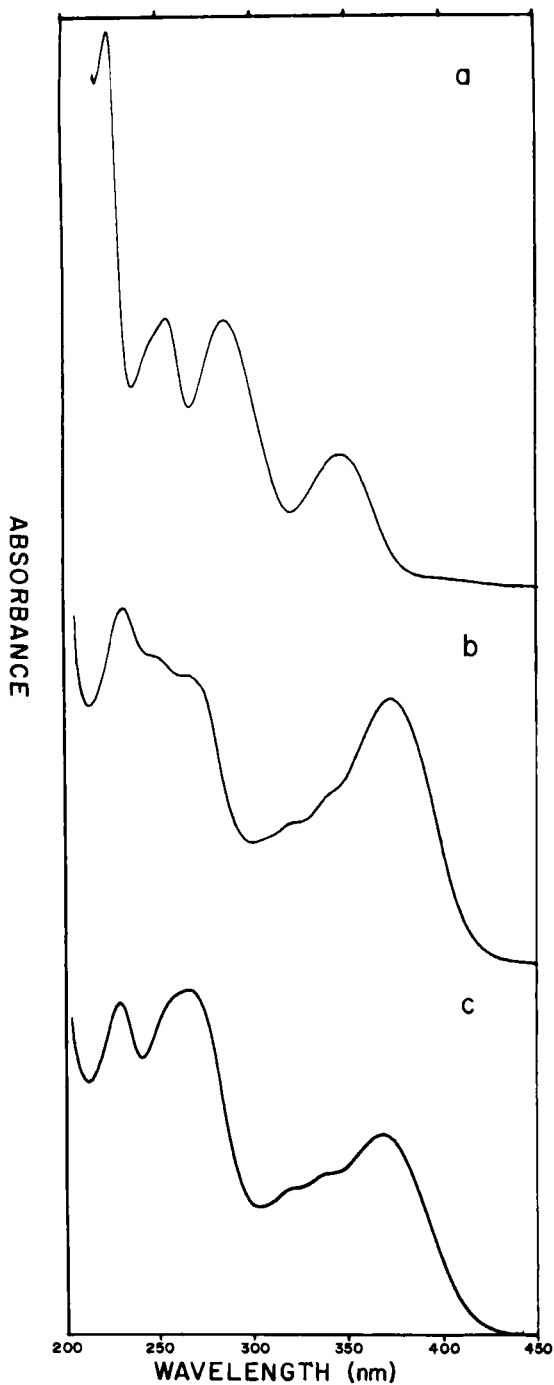


Figure 5. Ultraviolet absorption spectra of CTC-HCl in a, 0.1N HCl; b, 0.1N NaOH; and c, methanol.

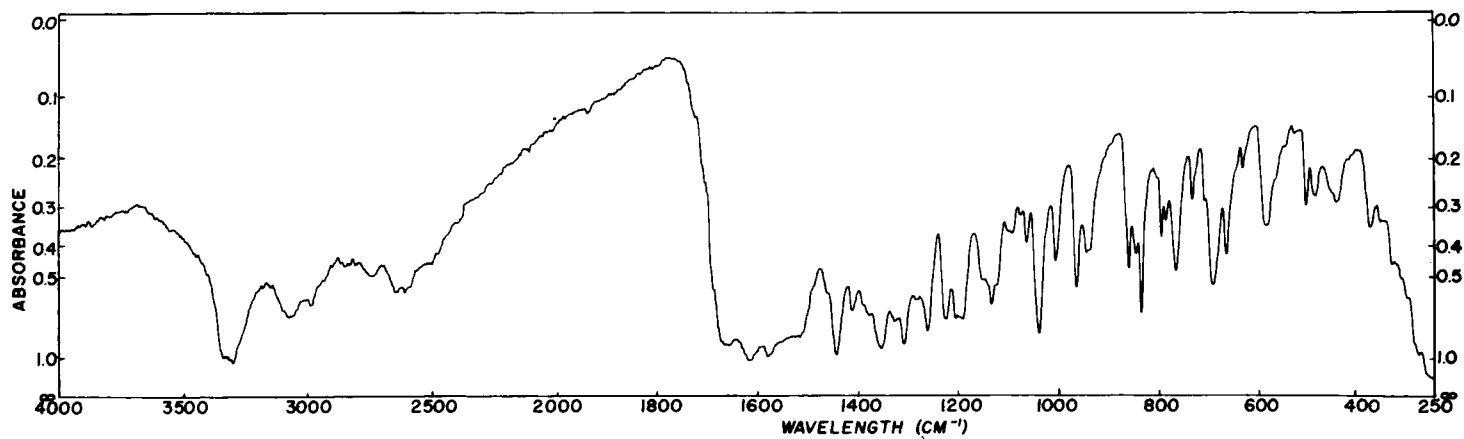


Figure 6. Infrared absorption spectrum of CTC-HCl as KCl pellet.

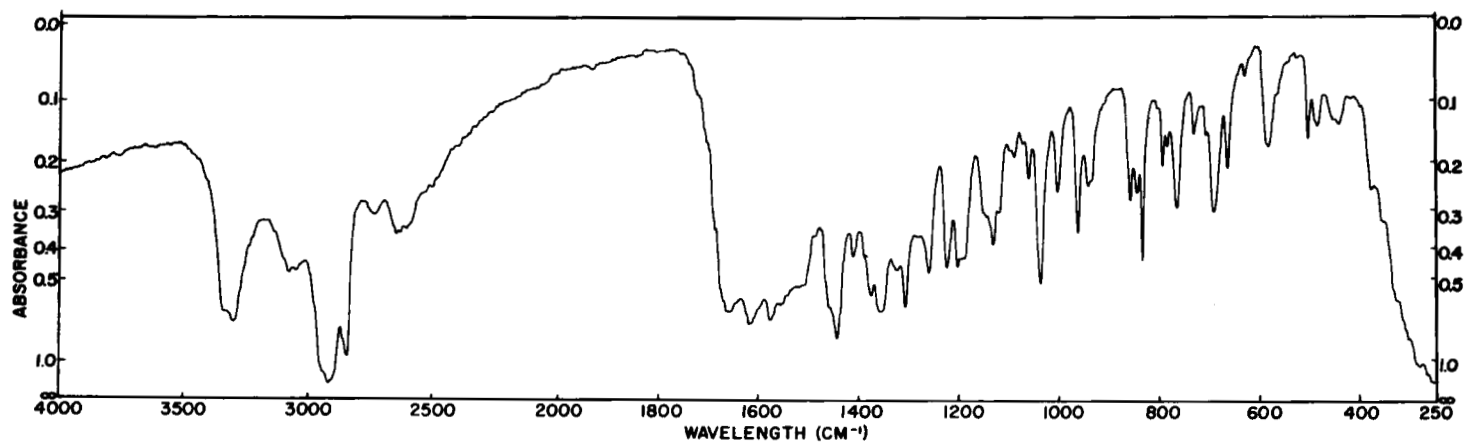


Figure 7. Infrared absorption spectrum of CTC-HCl as Nujol mull.

3.3 Spectropolarimetry

Because configurational information can be derived from optical rotatory dispersion and circular dichroism scans, considerable work has been conducted using these techniques to study the tetracyclines (32). The absolute configuration of CTC was determined using optical rotatory dispersion data (33). Spectral curves are presented in Figures 8 and 9. The circular dichroism spectrum is similar to that presented by Mitscher et al. (34), except that the values differ by a factor of about 1.5. In Table 3, data obtained by Mitscher and in FDA laboratories are compared.

3.4 Fluorescence

The CTC-HCl FDA Working Standard gives a yellow fluorescence under longwave ultraviolet (UV) light (375 nm) (35). The excitation (324 and 357 nm) and emission (443 nm) spectra of this standard dissolved in 0.05N NaOH (11.4 mg/50 ml) are presented in Figure 10.

TABLE 3
Molecular Ellipticities of CTC

Wavelength (nm)	Mitscher's Value ^a (X 10 ³)	FDA Value (X 10 ³)	Ratio
236	+10	+23	0.43
254	-31	-48	0.64
288	+48	+88	0.54
318	-24	-32	0.73
355(sh)	-14	-12	1.09

^aThese values were obtained from the graph in Reference 34.

4. SPECTRAL PROPERTIES (OTHER)

4.1 Proton NMR

The 60 MHz NMR spectra of CTC-HCl in DMSO-d₆ and in methanol-d₄ are shown in Figures 11 and 12, respectively. The sharp signals due to the methyl groups are readily assignable; their chemical shifts and intensities are consistent with the structure. The 2 methylene protons at C-5 are not equivalent and give two sets of multiplets. Only one multiplet is assignable in the methanol spectrum: the signal centered at 2.20 ppm. In DMSO-d₆ these protons appear between 1.5 and 2.3 ppm. The only methine proton actually assigned is the one at C-4. The close proximity to the positively charged N causes a downfield shift away from the other signals. When D₂O is added to the DMSO-d₆ solution, the H-4 signal remains constant. However, the two amide protons, originally at 9.15 and 9.55 ppm, exchange with the deuterium and are no longer observed. These signals were not observed in methanol-d₄, again, due to

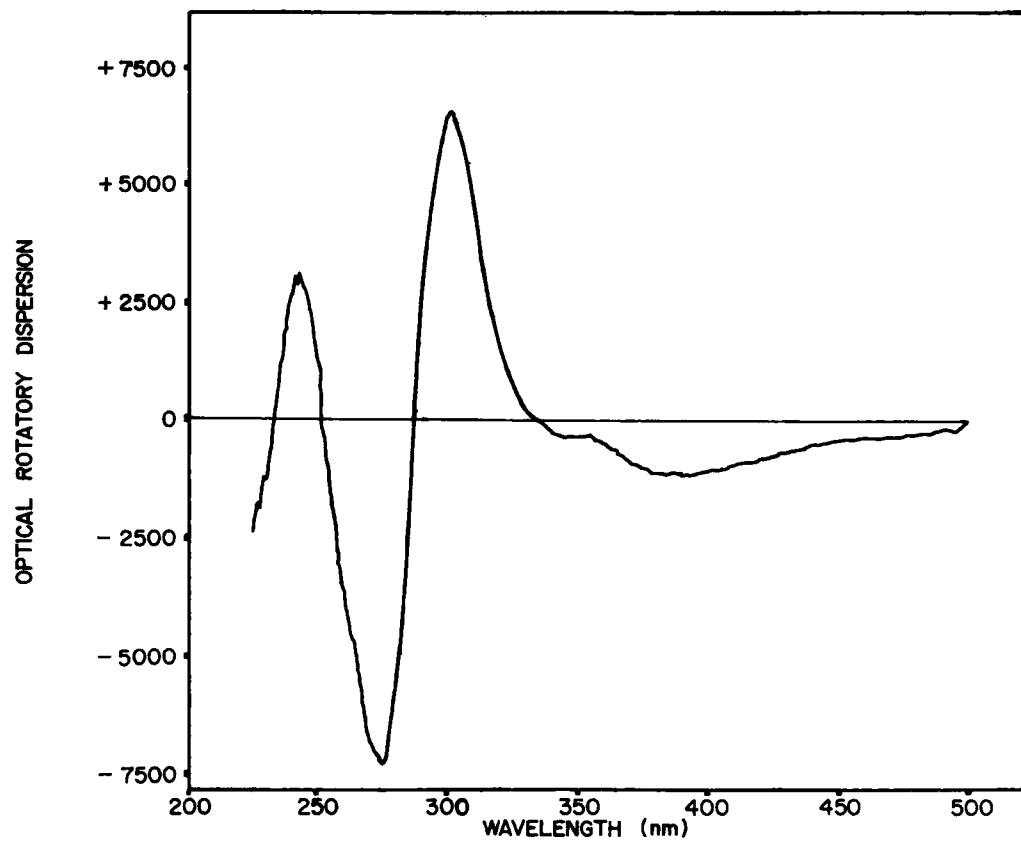


Figure 8. Optical rotatory dispersion spectrum of CTC-HCl.

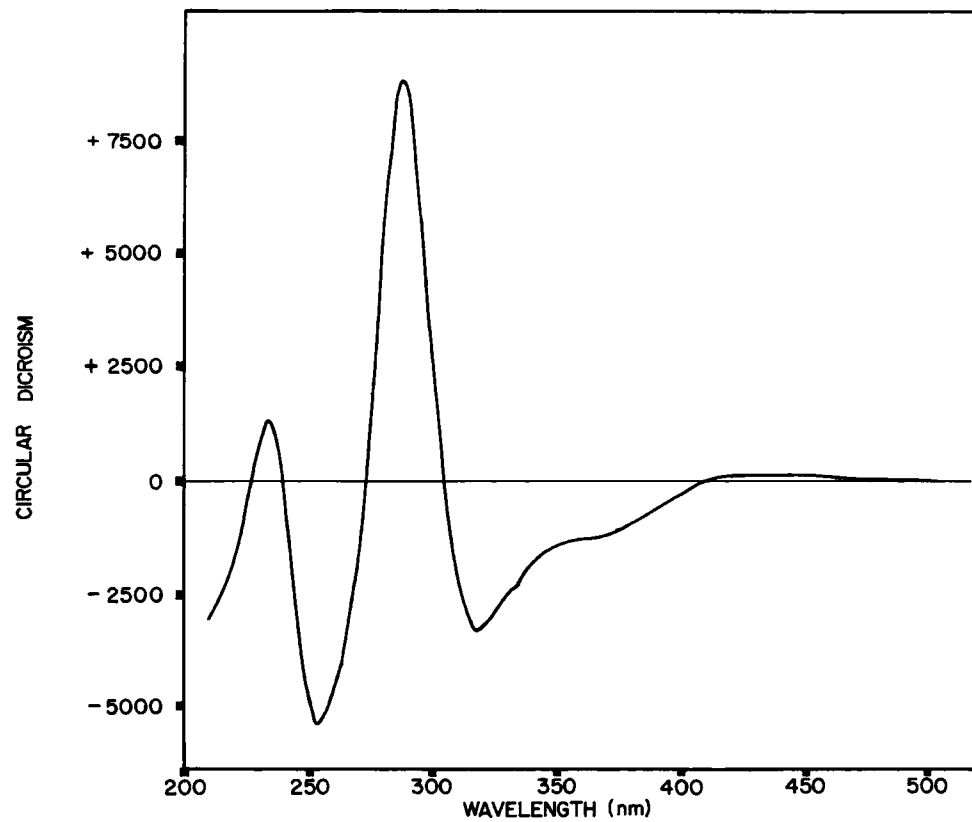


Figure 9. Circular dichroism spectrum of CTC-HCl.

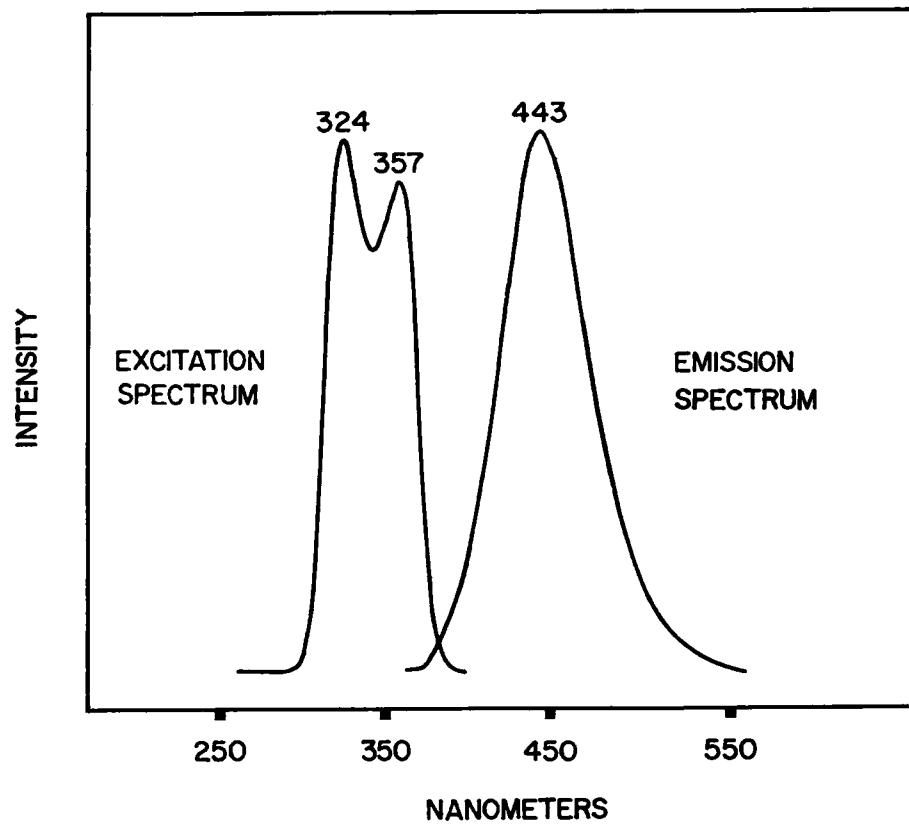


Figure 10. Excitation and emission fluorescence spectra of CTC-HCl.

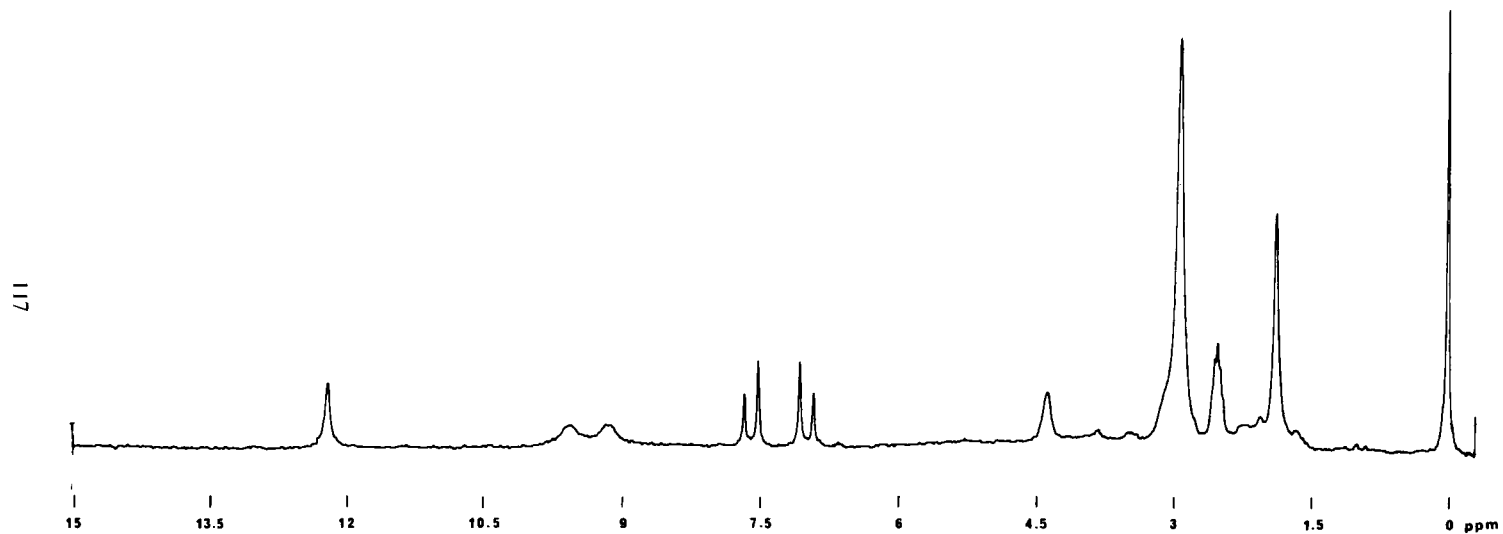


Figure 11. Proton magnetic resonance spectrum of CTC-HCl in DMSO-d₆.

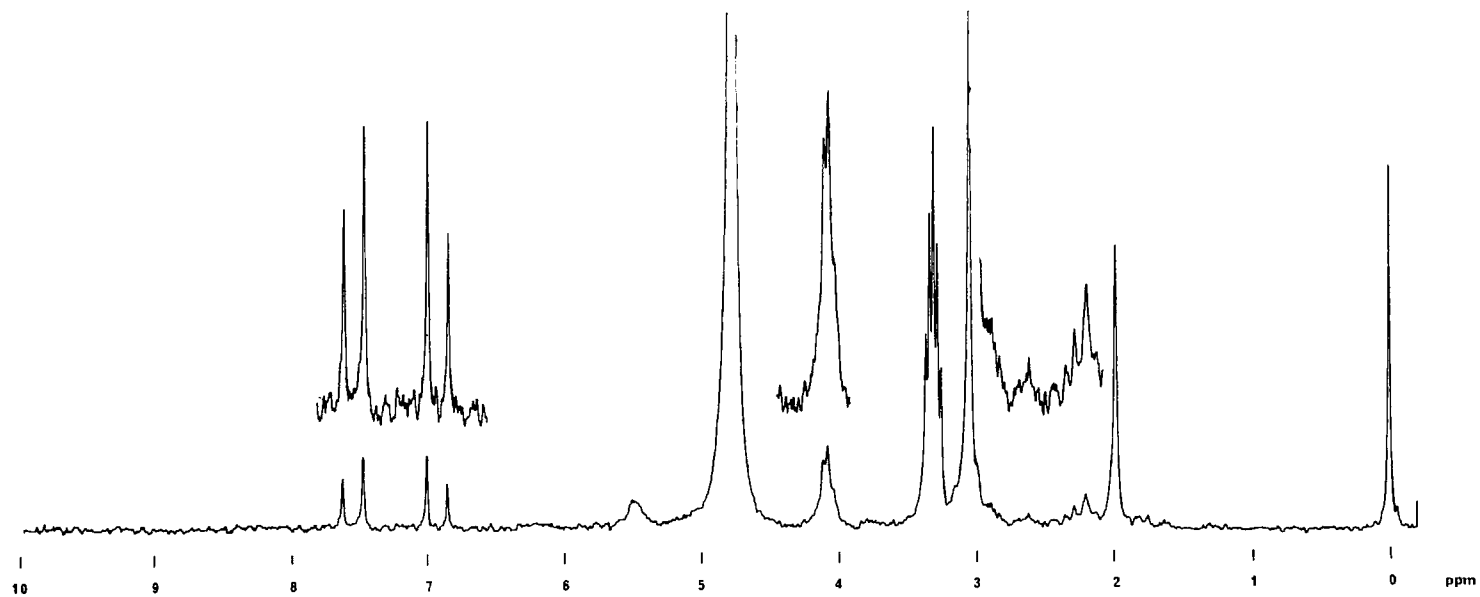


Figure 12. Proton magnetic resonance spectrum of CTC-HCl in methanol- d_4 .

deuterium exchange. Data from the literature (36), based on a spectrum obtained in DMSO- d_6 , are included in Table 4. As can be seen from the table, these values are generally upfield from our results. Although these differences were unexpected, no explanation will be attempted here (37).

TABLE 4
Chemical Shifts^a

Proton	Literature (36)	Solvent	
		DMSO- d_6	Methanol- d_4
C ₆ -CH ₃	1.7	1.88 (s)	1.98 (s)
C ₄ -N(CH ₃) ₂	2.7	2.93 (s)	3.05 (s)
H-8	6.4 - 7.0	7.58 (d)	7.55 (d)
H-9	6.4 - 7.0	6.98 (d)	6.93 (d)
H-4	4.0	4.36 (br. s)	4.10 (d)
H-5	1.7 - 2.1	1.5 - 2.3 (m,s)	2.20 (m) ^b
C ₂ -CONH ₂	8.4, 8.9	9.15, 9.55	not observed ^c
H-4a	2.7	not assigned ^d	not assigned ^d
H-5a	2.7	not assigned ^d	not assigned ^d

^aSpectra were obtained on a Perkin-Elmer R-12B equipped with a Nicolet TT-7 Fourier transform accessory. Chemical shifts are reported in ppm downfield from internal TMS. Multiplicities are indicated as s = singlet, d = doublet, and m = multiplet.

^bThis multiplet represents one of the H-5 protons. The chemical shift of the other one was not assigned.

^cThese protons exchange with the deuterium of the solvent and thus are not observed.

^dThe chemical shifts of these protons are not assigned. They are obscured by the -N(CH₃)₂ and/or the solvent.

4.2 ¹³C NMR

Proton-noise decoupled and single-frequency off-resonance decoupled carbon-13 NMR spectra were determined for the CTC Working Standard (Figure 13).

The observed chemical shifts of the 22 carbon atoms compare closely with those reported by Frank (38). The only significant difference observed was that of the dimethylamino carbons which were found at 48.5 ppm rather than at Frank's reported value of 41 ppm. This difference in chemical shifts can probably be ascribed to differing amounts of water present in the DMSO- d_6 solvents and hence in the pH of these respective solutions.

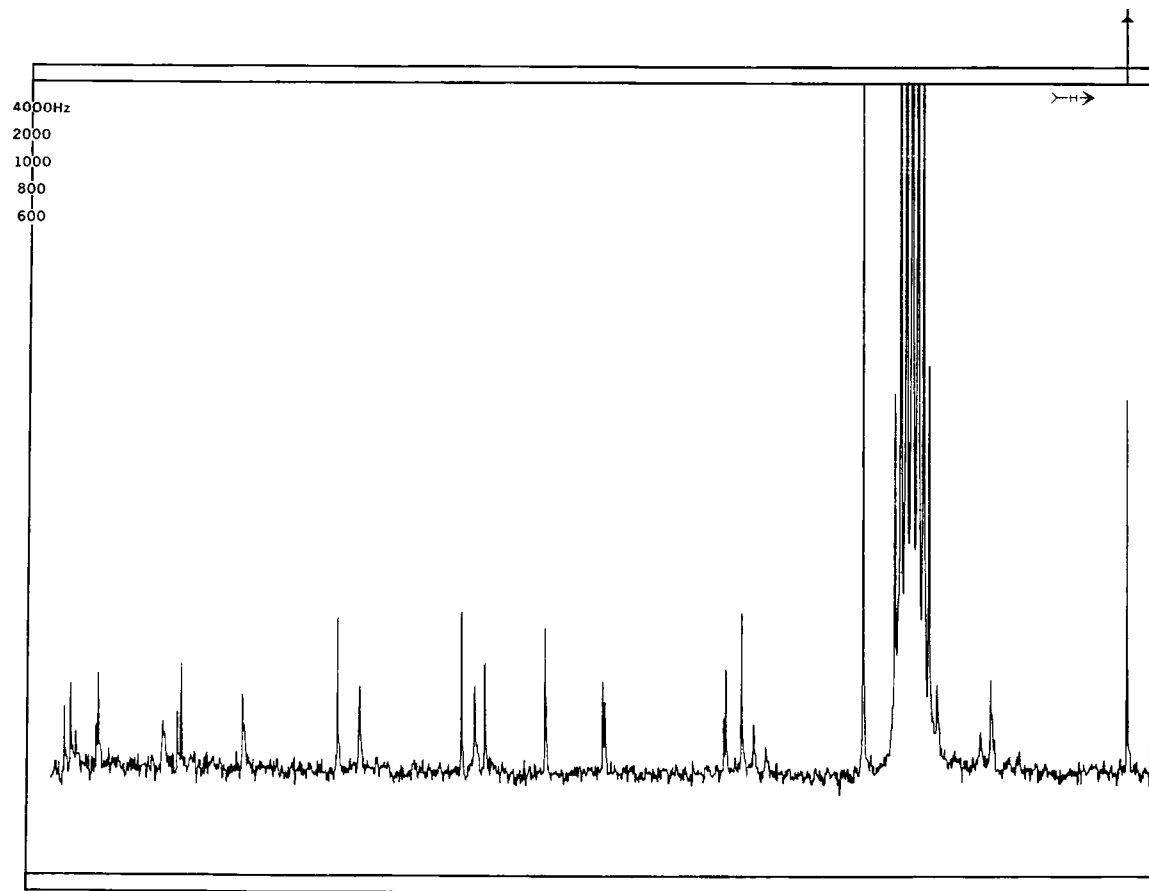


Figure 13. ^{13}C NMR spectrum of CTC-HCl.

Spectra were determined using a pulse width of 4 μ seconds, which corresponds to a flip angle of 18° and a 1 second pulse delay time. The 4000 Hz spectrum was described using 8192 data points.

The observed resonance lines of CTC-HCl (39) and their assignments are shown in Table 5.

TABLE 5
Observed Resonance Lines of CTC-HCl and Their Assignments

Carbon Assignment	Chemical Shift ^a
CH ₃	25.1 q
C-5	27.0 t
C-4a	34.9 d
C-5a	42.0 d
N(CH ₃) ₂	48.5 q
C-4	68.3 d
C-6	70.4
C-12a	73.3
C-2	95.5
C-11a	106.1
C-10a	117.0
C-9	118.9 d
C-7	121.3
C-8	139.7 d
C-6a	143.6
C-10	160.8
CONH ₂	172.0
C-12	175.3
C-3	187.1
C-1	192.1
C-11	193.3

^aIn DMSO-d₆, parts per million from TMS (0.00 ppm). q = quadruplet, t = triplet, d = doublet.

4.3 Mass Spectrometry

Mass spectral studies, including low voltage techniques and accurate mass measurements, have been reported by Hoffman (40) on tetracycline and eight related compounds. As a result of good spectral correlations among these compounds, the major fragmentation processes are discussed in terms of two compounds in the series: 5 α ,6-anhydrotetracycline and dedimethylaminotetracycline, with only a selected number of ions reported for CTC. Further studies on these antibiotics, including CTC, were conducted by Morris and Cairns (41). However, little information on their results is included in the extended abstract of the meeting, and a full report has not

been published. Personal communications with the authors indicate that the results of their work are in close agreement with data reported here.

The fragmentation pattern of CTC can be discussed in terms of three different mechanisms. These are:

a. Processes resulting from the capacity of the dimethylamino group to direct losses of small fragments together with its ability to retain and stabilize the positive charge.

b. Fragmentation of the D ring system with charge retention on the fused ring portion of the molecule.

c. Fragmentation of the D ring system with charge retention elsewhere than on the fused ring moiety.

The mass spectrum of CTC, shown in Figure 14 (42), is characterized by a reasonably intense molecular ion at m/e 478 with the concomitant isotope peak at $P+2$ representing one chlorine atom in the ring system. Although it has been suggested that this chlorine atom be employed as a tracer via the ^{37}Cl isotope ratio for detection of species containing the A ring and adjunct ring systems, many of these ions are of such low relative intensity that this would have only limited usefulness toward that end, except perhaps at higher mass values.

The dominance of the fragmentation processes by the presence of the 4-dimethylamino group gives rise to the intense ions at m/e 44, m/e 58 $(\text{CH}_3)_2\text{-}\overset{+}{\text{N}}=\text{CH}_2$, m/e 71 $(\text{CH}_3)_2\text{-}\overset{+}{\text{N}}\text{-CH=CH}_2$, and m/e 84 $(\text{CH}_3)_2\text{-}\overset{+}{\text{N}}\text{=CH-CH=CH}_2$. The ion at m/e 98 ($\text{C}_5\text{H}_8\text{NO}^+$) appears to involve a cyclization of the dimethylamino group with elements of the D ring. Of particular note is the loss of 43 atomic mass units from the molecular ion to give the intense ion at m/e 435 and from the ion at m/e 443 to yield m/e 400 (Figure 15).

The presence of the amide functional group is indicated by observations of successive losses of NH_3 and OH (as water) from the molecular ion to yield ultimately the ion at m/e 443. The loss of water at m/e 460 appears to be slightly favored over the competing loss of NH_3 at m/e 462 from the abundances of the respective ions.

Fragmentation of the D ring can provide the dual purpose of determining the substituent at the 4 position (dimethylamino in the case of CTC) and/or help to locate functionalities elsewhere in the ring system. This, however, requires a detailed examination of analogs such as in the work of Hoffman (40).

The ion at m/e 365 will locate the substituents at the 2 and 3 positions of the D ring by determination of the mass of the lost substituent and also give evidence concerning the extent of substitution on the A, B, and C rings. This

SPEC. NO. 20039 LS CHLORTETRACYCLINE HCL

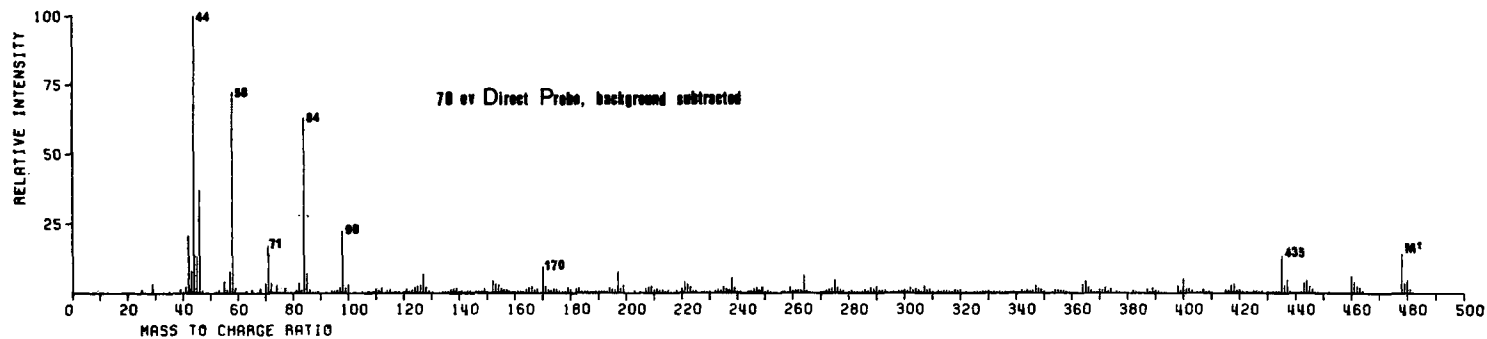


Figure 14. Electron impact mass spectrum of CTC-HCl. Instrument: Varian MAT 311; source temperature sufficient to produce vaporization. (Chemical ionization using ammonia as reagent gas gave MH^+ as base peak.)

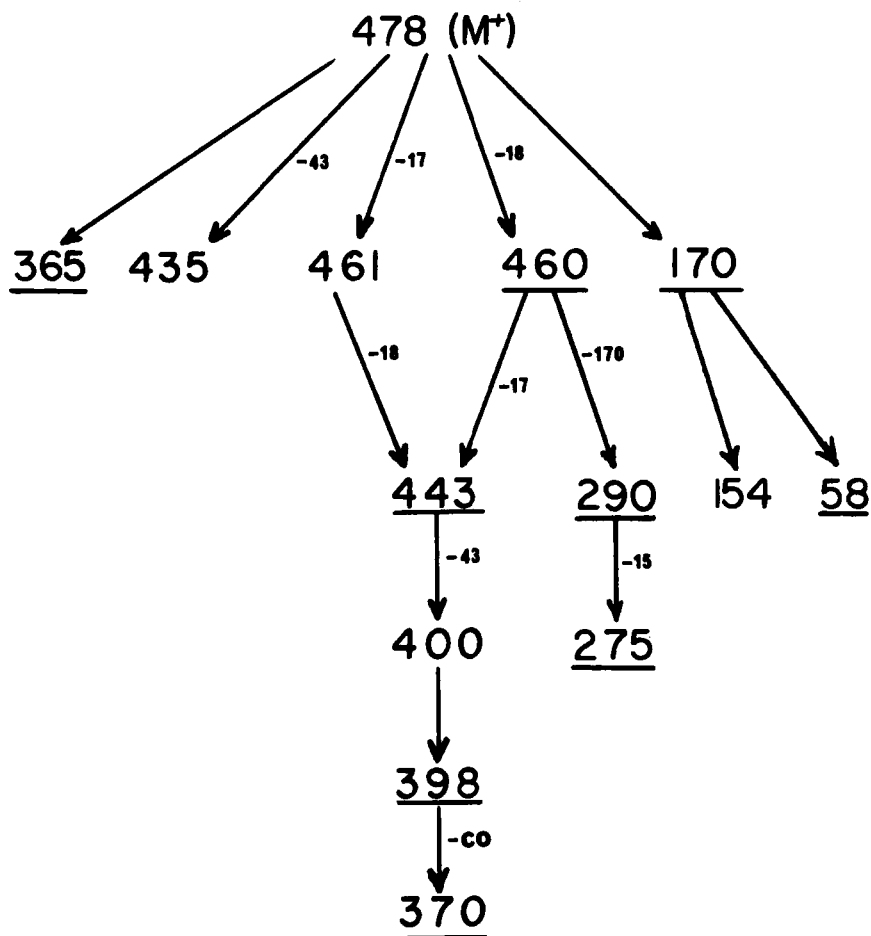


Figure 15. Major fragmentation pathways of CTC involving molecular ion and other significant high mass ions. Underlined m/e values indicate confirmation by accurate mass measurements.

knowledge plus the observed shift of the ion at m/e 170 should suffice to allow postulation of the substitution (Figure 16).

5. CHROMATOGRAPHY

5.1 Paper and Thin Layer

Some of the early reports on the chromatography of the tetracycline antibiotics prior to 1957 are of limited value. Fischbach and Levine (43) described a continuous ascending technique and Berti and Cima (44) reported an ascending method using aqueous sodium arsenite as the mobile solvent. Other authors (45,46) reported descending techniques and bioautographic means for locating the zones of activity.

All of these methods fail to show the presence of the epimeric form of the tetracyclines and in most instances streaking of the spots is a problem. A basic improvement in the paper chromatography of these antibiotics was achieved by Selzer and Wright (47) and Kelly and Bryske (48) when they reported methods for the pretreatment of the paper with complexing agents to bind the metallic ions which may be present.

The tetracyclines are well known for their ability to form complexes with polyvalent cations. This property changes their solubility characteristics in the mobile solvents and often results in troublesome streaking. To overcome this difficulty, Selzer and Wright used paper dipped in McIlvaine's buffer (pH 3.5) which contains citrate ions capable of binding the metallic ions. The chromatograms were developed with a mixture of nitromethane, chloroform, and pyridine (20:10:3) on paper still damp from the treatment with the buffer solution.

For the same purpose, Kelly and Bryske used paper impregnated with 0.1N disodium ethylenediaminetetraacetate (EDTA) and two mobile solvents: the organic phase from a mixture of *n*-butanol, ammonia, water (4:1:5) and the organic phase from a mixture of *n*-butanol, acetic acid, water (4:1:5). Disodium EDTA (0.1N) works as well as McIlvaine's buffer when it is used to treat the paper in the method of Walton et al. (49). A circular paper chromatographic method also using paper dampened with McIlvaine's buffer (pH 4.5) was reported by Urx et al. (50). They used a mixture of chloroform and *n*-butanol (4:1) as the mobile solvent.

Most of the methods in which the paper is treated with a chelating agent are capable of showing a separation of some of the tetracycline drugs from each other and from their respective epimeric forms. They are also capable of revealing the presence of common degradation compounds of these drugs.

The usual method of detecting chromatographed tetracycline antibiotics involves fuming the paper with ammonia vapor and observing the yellow fluorescence under UV light. As little as 0.2-0.5 μ g can be visualized by this technique.

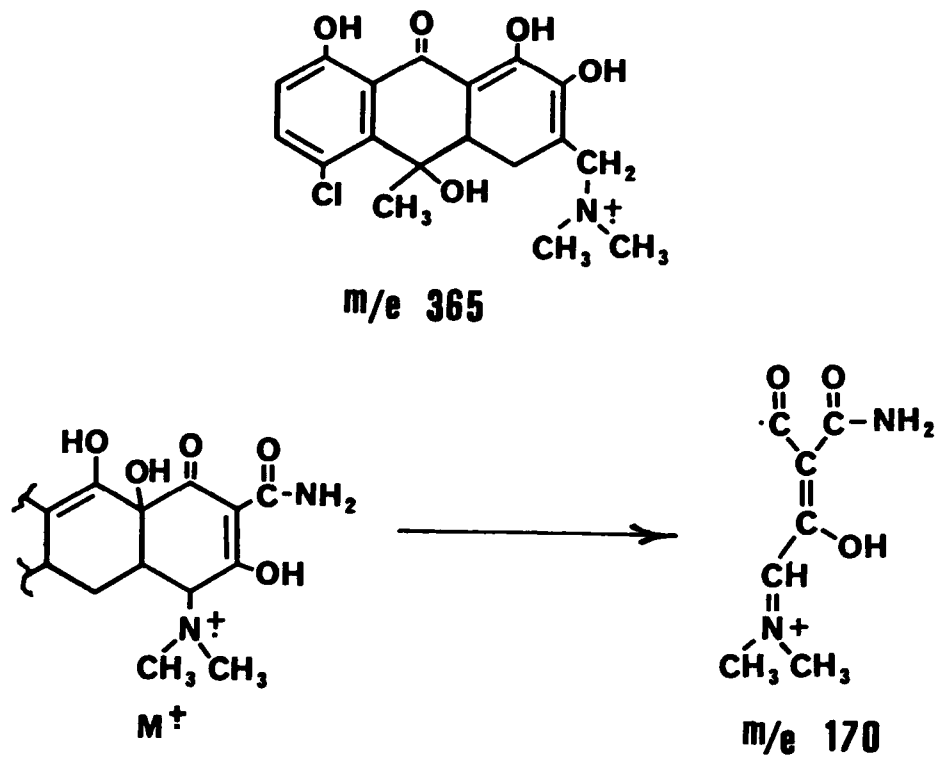


Figure 16. Proposed structures for ions at m/e 365 and m/e 170 for use in determination of ring substituents.

Some of the features that are successful for the chromatography of the tetracyclines on paper have been adapted to thin layer chromatography (TLC). Complexing agents are almost always used in the preparation of the plates and, in addition, several methods for hydrating the plates by the addition of glycerin and/or polyethylene glycol have been reported. Several investigators find it desirable to acid-wash the inorganic support in order to remove metallic cations. These TLC methods are much more time-consuming than most of the paper chromatographic procedures because of the special care often required to prepare and store the TLC plates. For this reason, where applicable, the paper chromatographic methods are preferable. However, for quantitative analysis of the tetracycline epimers and degradation products, TLC is usually considered to be better than paper chromatography.

In 1964, Somanini and Anker (51) described a method using Kieselguhr layers impregnated with a buffer solution containing glycerin. Nishimoto et al. (52) reported the use of silica gel layers treated with EDTA. In 1967, Ascione et al. (53) published a method using layers made from acid-washed diatomaceous earth. They prepared plates containing 0.1N EDTA, glycerin, and polyethylene glycol 400. Other investigators (54,55) have described methods which are modifications of improvements of the methods previously published.

5.2 Gas and Liquid Chromatography

Investigators have found it quite difficult to chromatograph the tetracyclines by gas-liquid techniques. Often, only fragments of the original sample are obtained (56). Tsuji and Robertson (57) did manage to chromatograph silylated CTC using 3% methyl silicone on Gas-Chrom Q and other stationary phases. However, with the advent of refined high pressure liquid chromatographic (HPLC) techniques, interest in gas chromatographic methods for the tetracyclines has diminished.

A number of papers have appeared reporting the HPLC separation of CTC from its isomers and/or other tetracyclines. There is not a consensus of opinion as to the most satisfactory approach; thus, it appears that at this time one must still verify the optimal system for a particular instrument. Methods found in the literature for CTC are described in Table 6. EDTA is added to prevent the formation of complexes of the tetracyclines with metallic surfaces.

Other chromatographic techniques that have been applied to the tetracyclines, including CTC, involve low pressure column chromatography. Ascione et al. (64) developed a semiautomated system whereby sample solutions are automatically injected onto a column of diatomaceous earth mixed with

TABLE 6
High Pressure Liquid Chromatography of CTC

Mobile Phase	Stationary Phase	Migration Time (min.)	Ref.
20% Methanol, 80% 0.05M ammonium carbonate, 0.02M EDTA	C8/Lichrosorb 10 μ m, 25 cm x 3.2 mm	34.5	58
Phosphate buffer in 13% methanol, 0.85 ml/min., pH 2.5	Zipax-hydrocarbon polymer, 2.1 x 1000 mm	6	59
Aqueous perchlorate-citrate buffer mixed with CH ₃ CN	Sil-X, 13 μ m, 5 x 125 mm	1.3	60
EDTA, NaCl in 30% methanol, NH ₃ , pH 9.9	Ion-X-SA, anion exchanger	3	61
EDTA, PO ₄ , isopropanol-water, pH 7.6, 2 ml/min.	μ Bondapak C ₁₈ , 300 x 4 mm	11.6	62
EDTA, NO ₃ , 9.6% ethanol, pH 9, 1 ml/min.	Zipak, 1.8 x 1000 mm	22	63

a solution of EDTA and polyethylene glycol. CTC is eluted with the organic phase of a mixture of chloroform, benzene, aqueous solution of EDTA, and polyethylene glycol. Ragazzi and Veronese (65) separated CTC from other tetracyclines by means of gel permeation chromatography.

6. MANUFACTURE

6.1 Fermentation

A medium (containing corn steep liquor; calcium carbonate; sucrose; ammonium, ferrous, manganese, and zinc sulfates; and ammonium, cobalt, and magnesium chlorides) is sterilized and diluted with water to the desired concentration. It is inoculated with Streptomyces aureofaciens, kept at 27°C, and aerated and agitated for ~60 hours, with lard oil added to control foaming (66).

6.2 Isolation

The mash from the Streptomyces aureofaciens fermentation broth is acidified and filtered. The filtrate is adjusted to the desired pH, usually 7-8.5, and various flocculating or chelating agents may be added (e.g., vinyl acetate-maleic anhydride copolymer, sodium EDTA, ammonium oxalate, Arquad). The precipitate is (1) stirred with filter aid, filtered, stirred with HCl, refiltered, mixed with 2-ethoxyethanol, filtered, washed, and the filtrates are combined, acidified with HCl, NaCl is added, and the crystals are collected, washed with 2-ethoxyethanol, water, and ethanol, and dried (67), or (2) extracted into methyl isobutyl ketone, the extracts are combined, filtered, and acidified with HCl, and the crystals are collected and washed with water, 2-ethoxyethanol, and isopropanol, and vacuum-dried. If the crystals are greenish, they are treated with sodium hydrosulfite at pH 1.8, filtered, washed, and dried as in (1) above (68).

7. STABILITY

CTC-HCl, as a dry powder, is a stable yellow crystalline material. The situation in aqueous solution, however, is quite different. In sodium hydroxide solutions, CTC is converted to iso-CTC on standing (69). The solution becomes colorless and exhibits a strong blue fluorescence under UV light. Dilute solutions of CTC, in pH 7.5 buffer, make the same conversion at 100°C.

In acid solutions, CTC is converted to anhydro-CTC (70). This change is greatly accelerated by heating and results in a yellow product that has a maximum absorbance at 445 nm.

In addition, CTC undergoes reversible epimerization of the 4-dimethylamino group (71). This occurs slowly in water or methanol but is hastened in buffer solutions in the range

of pH 2-6. The rate of epimerization is undetectable in solutions more acidic than pH 2 (72). The antimicrobial activity of the epimer is probably zero. The slight activity found for this material is probably due to the reemergence of CTC under the test conditions.

In a fashion analogous to that of CTC, its epimer can be converted to epianhydro-CTC in acid solution.

8. ANALYTICAL METHODS

8.1 Microbiological

The microbiological methods used for the determination of CTC potency in body tissues and fluids, bulk products, and pharmaceutical formulations can be separated into two testing procedures: (1) agar diffusion plate method (cylinder-plate) and (2) turbidimetric method.

1. Agar diffusion plate method (cylinder-plate):

This method is employed for determining the potency of CTC in human and animal pharmaceutical formulations, bulk products, serum, tissues, urine, dairy products, and animal feeds. The cylinder-plate procedure is described by Grove and Randall (73) and the Code of Federal Regulations (74). Additional methods using this assay are described by Kramer et al. (75). The official final action method for CTC assay in animal feeds is described in the Association of Official Analytical Chemists' Official Methods of Analysis (76).

2. Turbidimetric method:

This method is used in lieu of the diffusion plate method for human and animal pharmaceutical formulations and bulk products. The turbidimetric method is described in the Code of Federal Regulations (77).

8.2 Chemical

The physical structure of CTC has provided a good source of characteristics useful for the analysis of this antibiotic. Although titrimetric (78) and polarographic (79) methods have been reported, the most useful procedures have been based on the spectroscopic properties of CTC and its derivatives (80).

In 1949, Levine et al. (81) published two procedures for the assay of CTC. One method was based on the conversion of CTC by heating in acid to the more intensely yellow anhydro-CTC derivative. The other method was based on measuring the blue fluorescence of iso-CTC, which was prepared by heating CTC in pH 7.5 phosphate buffer.

Others have described modifications of these methods for various purposes. Hiscox (82) suggested the direct spectrophotometric assay of CTC in either acid or alkaline solution at various UV wavelengths. The possible contamination of CTC with other tetracycline drugs was addressed by Chicearelli et

al. (83). They corrected for the possible presence of tetracycline in CTC using the fact that the former is unchanged in dilute alkali while CTC is converted to the colorless iso-CTC.

The tetracycline which may be present is then converted to an anhydro derivative by heating in acid and is measured spectrophotometrically. Feldman et al. (84) developed the alkaline degradation method to measure CTC in fermentation mash and Spock and Katz (85) used this method to determine CTC in animal feed premixes.

The natural fluorescence of CTC and its derivatives has been used extensively to determine small amounts of CTC in biological materials. Kohn (86) showed that the fluorescent complex formed by CTC with calcium ions and barbital could be extracted from animal tissues into an organic solvent and then measured spectrofluorometrically. The intense fluorescence of anhydro-CTC was used by Hayes and DuBuy (87) to determine CTC in animal tissues, tissue culture cells, and bacteria. Poiger and Schlatter (88) extracted CTC from biological material into ethyl acetate as the CTC-calcium trichloroacetate ion pair. The fluorescence of the antibiotic was then enhanced by the addition of magnesium ions and a base.

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DOBUTAMINE HYDROCHLORIDE

Rafik H. Bishara and Harlan B. Long

1. Description
 - 1.1 Nomenclature
 - 1.1.1 Chemical Name
 - 1.1.2 Nonproprietary Name
 - 1.1.3 Proprietary Name
 - 1.2 Formula
 - 1.2.1 Empirical
 - 1.2.2 Structural
 - 1.3 Molecular Weight
 - 1.4 Appearance, Color, Odor, and Taste
2. Physical Properties
 - 2.1 Melting Range
 - 2.2 Simple Solubility Profile
 - 2.3 pH Range
 - 2.4 Dissociation Constant (pKa)
 - 2.5 Thermal Analyses
 - 2.5.1 Differential Thermal Analysis
 - 2.5.2 Thermogravimetric Analysis
 - 2.6 Crystallinity
 - 2.6.1 Crystalline Habit
 - 2.6.2 X-Ray Powder Diffraction
 - 2.7 Ultraviolet Spectrum
 - 2.8 Infrared Spectrum
 - 2.9 Nuclear Magnetic Resonance Spectrum
 - 2.10 Mass Spectrum
3. Synthesis
4. Stability—Degradation
5. Absorption, Metabolism, and Excretion
 - 5.1 In Dog
 - 5.2 In Man
6. Methods of Analysis
 - 6.1 Elemental Analysis
 - 6.2 Chloride Identity
 - 6.3 Nonaqueous Titration
 - 6.4 Chloride Determination
 - 6.5 Chromatography
 - 6.5.1 Thin-Layer Chromatography
 - 6.5.2 Gas Chromatography
 - 6.5.3 High Performance Liquid Chromatography
7. Analysis of Biological Samples
 - 7.1 Enzymatic Assay
 - 7.2 Chromatographic Assays
 - 7.2.1 Thin-Layer Chromatography
 - 7.2.2 Gas Chromatography
 - 7.2.3 High Performance Liquid Chromatography
 - 7.3 Mass Spectrometry (GC/MS)

8. Analysis of Pharmaceutical Formulations
 - 8.1 Chromatographic Assays
 - 8.1.1 Thin-Layer Chromatography
 - 8.1.2 Gas Chromatography
 - 8.1.3 High Performance Liquid Chromatography
 - 8.2 Spectrophotometric (UV)
9. Acknowledgments
11. References

1. Description

1.1 Nomenclature

1.1.1 Chemical Name

(±)-4-[2-[[3-(4-Hydroxyphenyl)-1-methylpropyl]amino]ethyl]-1,2-benzenediol, hydrochloride

1.1.2 Nonproprietary Name

Dobutamine hydrochloride

1.1.3 Proprietary Name

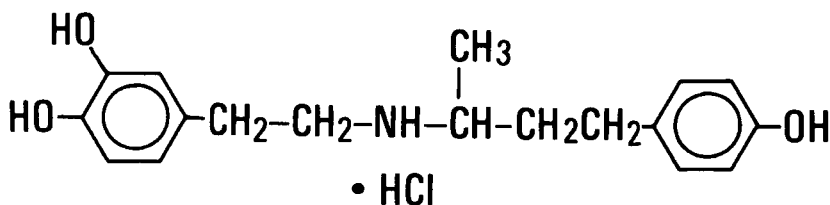
DOBUTREX[®]

1.2 Formula

1.2.1 Empirical

$C_{18}H_{23}NO_3 \cdot HCl$

1.2.2 Structural



1.3 Molecular Weight

337.85

1.4 Appearance, Color, Odor, and Taste

White to off-white, odorless powder with a slightly bitter taste.

2. Physical Properties

2.1 Melting Range

189 - 191°C

2.2 Simple Solubility Profile

The sample is sonicated for one minute at ambient temperature.

<u>Solvent</u>	<u>mg/ml</u>
Water	>3.33
pH 1.2 (USP XIX)	>2.50
pH 4.5 (USP XIX)	>3.33
pH 7.0 (USP XIX)	>3.33
Ethanol	>5.00
Methanol	>10.00
Pyridine	>5.00
Octanol	<0.50
Diethyl ether	<0.50
Ethyl acetate	<0.50
Chloroform	<0.50
Benzene	<0.50
Cyclohexane	<0.50

2.3 pH Range

The pH of a 5% w/v solution in a water/ethanol (1:1) solution is about 4.9.

2.4 Dissociation Constant

The pKa in dimethylformamide/water (66:34) is 9.45.

2.5 Thermal Analyses

2.5.1 Differential Thermal Analysis

A DTA thermogram of dobutamine hydrochloride, at a heating rate of 5°C per min. in a nitrogen atmosphere of 40 cc per min., shows (figure 1) an endotherm at 196°C which appears to indicate a melt.

2.5.2. Thermogravimetric Analysis

A TGA thermogram of dobutamine hydrochloride, run simultaneously with the DTA, shows (figure 1) no weight loss until 233°C which results from decomposition.

2.6 Crystallinity

2.6.1 Crystalline Habit

Dobutamine hydrochloride generally crystallizes in a random manner usually from an oil (1). This results in a nondescript crystalline formation. In only few cases does the drug exhibit any crystalline habit of interest. Upon careful and patient crystallization small thin plates and/or small needles are formed.

2.6.2 X-Ray Powder Diffraction

The following data describe the pattern for dobutamine hydrochloride, where d is equal to the interplanar spacing measured in terms of Angstroms (Å). The ratio I/I_1 is the intensity of the X-ray maxima based upon a value of 100 for the strongest line. A b indicates a broad line resulting from failure to resolve two closely spaced diffraction maxima.

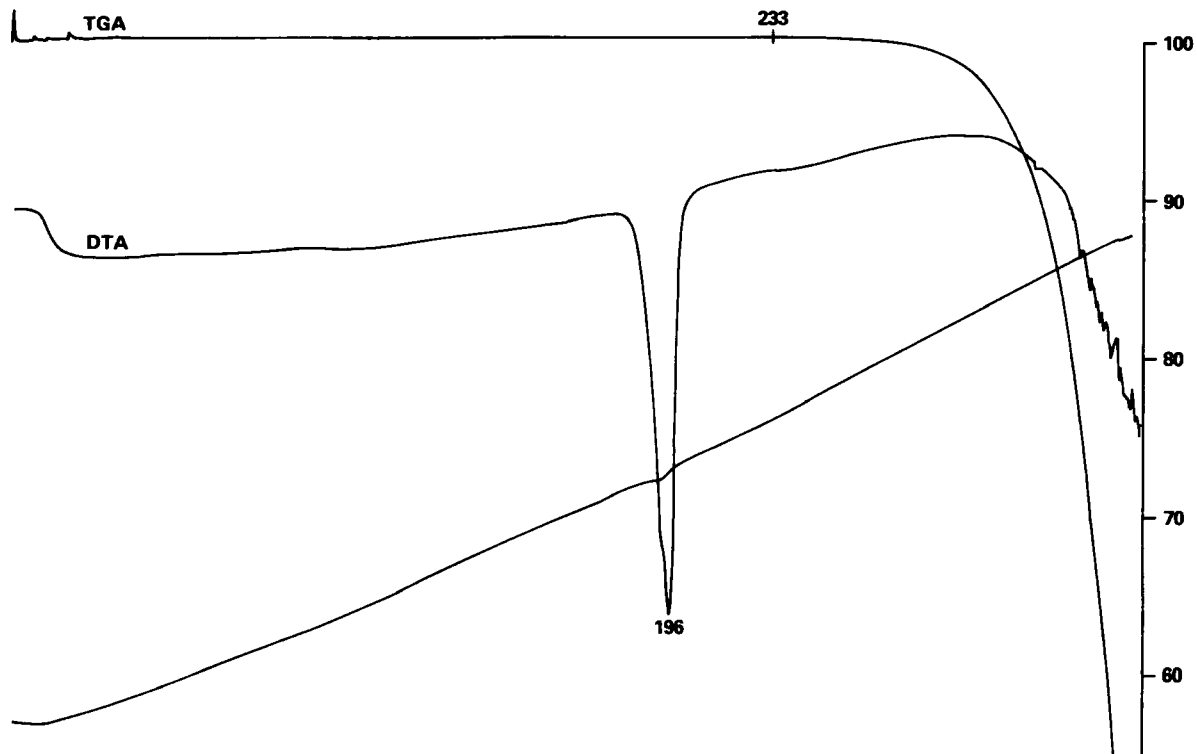


Figure 1. Thermogravimetric Analysis and Differential Thermal Analysis Thermograms of Dobutamine Hydrochloride

Cu-Ni- λ 1.5418 \AA

<u>d</u>	<u>I/I₁</u>	<u>d</u>	<u>I/I₁</u>
9.07	7	2.74	14
6.87	5	2.63	9
6.37	7	2.51	16
5.27	18b	2.40	5
4.98	18	2.35	2
4.50	100	2.27	4
4.13	16	2.22	2
4.04	61	2.14	7
3.78	45	2.11	4
3.60	45	2.06	5
3.44	16b	2.01	7
3.18	18	1.99	7
3.11	7	1.95	7
3.01	14		
2.86	7		

2.7 Ultraviolet Spectrum

The ultraviolet spectrum of dobutamine hydrochloride in methanol is given in figure 2. The spectrum exhibits maxima at 281 and 223 nm with molar absorptivities of 4,768 and 14,400, respectively. When aqueous potassium hydroxide is added to the methanolic solution of dobutamine hydrochloride the maxima at 281 and 223 shift to 293 ($\epsilon=6,100$) and 240 nm, respectively. These shifts are reversible by addition of hydrochloric acid. When the absorption spectrum of the drug is recorded in water rather than methanol, slight shifts in peak positions and intensities are observed:

$$\begin{aligned}\lambda_{\text{max}} &= 278 \text{ nm } (\epsilon = 4,112) \\ \lambda_{\text{max}} &= 220 \text{ nm } (\epsilon = 13,500).\end{aligned}$$

2.8 Infrared Spectrum

The infrared spectrum of dobutamine hydrochloride in a potassium bromide disk is given in figure 3. Major band assignments are as follows:

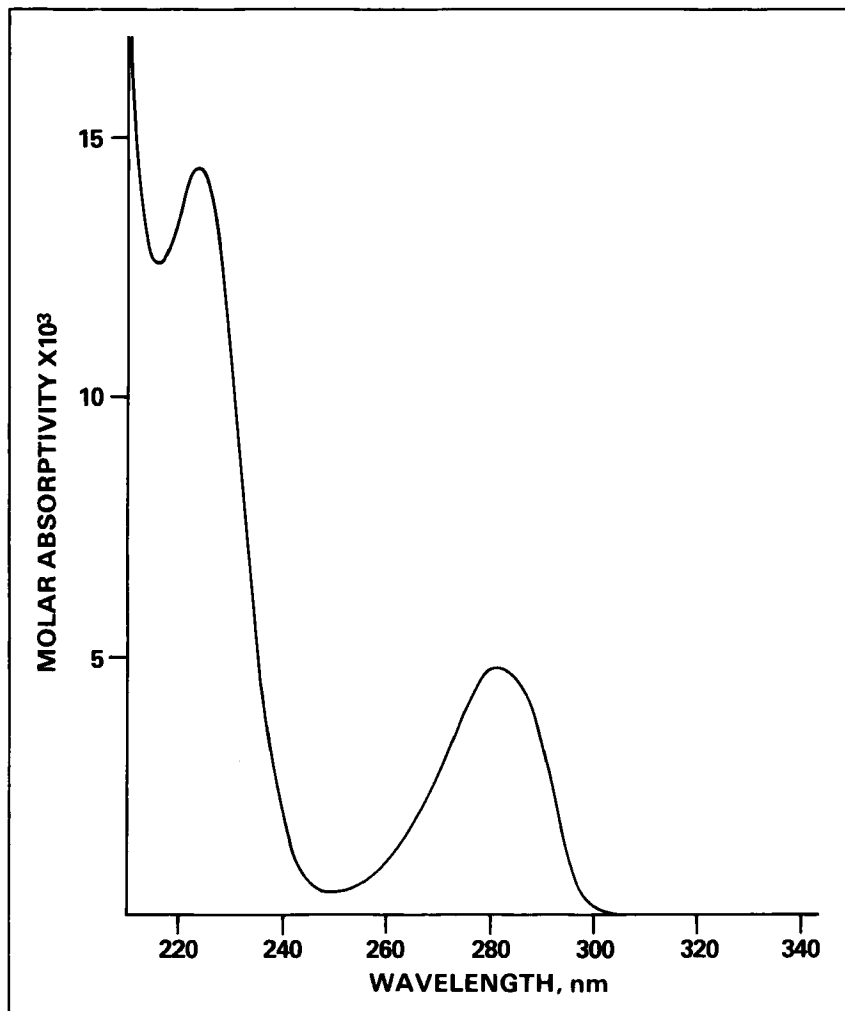


Figure 2. Ultraviolet Spectrum of Dobutamine Hydrochloride

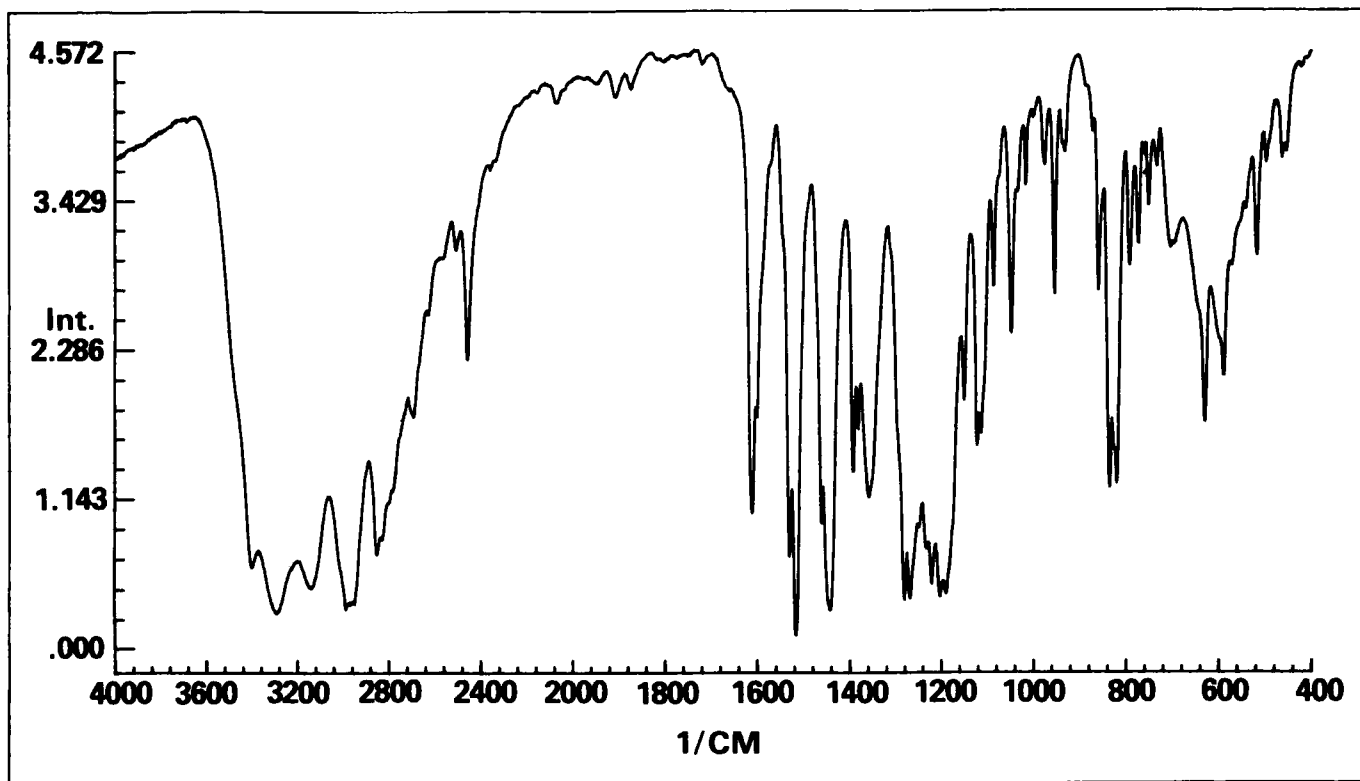
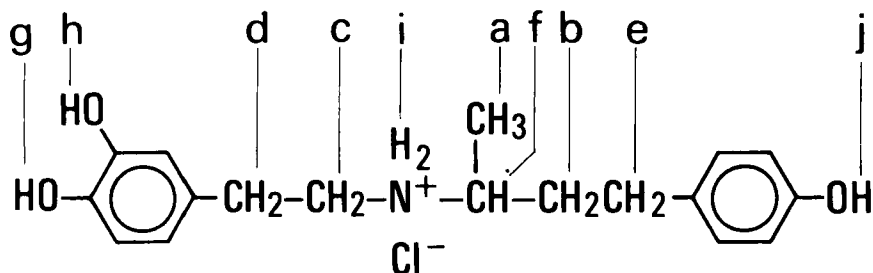


Figure 3. Infrared Spectrum of Dobutamine Hydrochloride

<u>Band Position, cm^{-1}</u>	<u>Assignment</u>
3400, 3300 and 3140	phenolic O-H stretching
2960 and 2840	C-H stretching (overlapping O-H bands)
2700 and 2450 (weak bands)	Mainly NH_2 , NH stretching
1610, 1530, 1520 and 1450	Aromatic ring stretching
1440, 1390 and 1380	CH_2 , CH_3 , C-H bending
1360, 1280-1190 (several bands)	phenolic C-O stretching
1150 and lower	Mainly skeletal and aromatic C-H bending

2.9 Nuclear Magnetic Resonance Spectrum

The 60 MHz proton NMR Spectrum of dobutamine hydrochloride in deuterated dimethylsulfoxide is given in figure 4. Assignments of the bands are as follows:



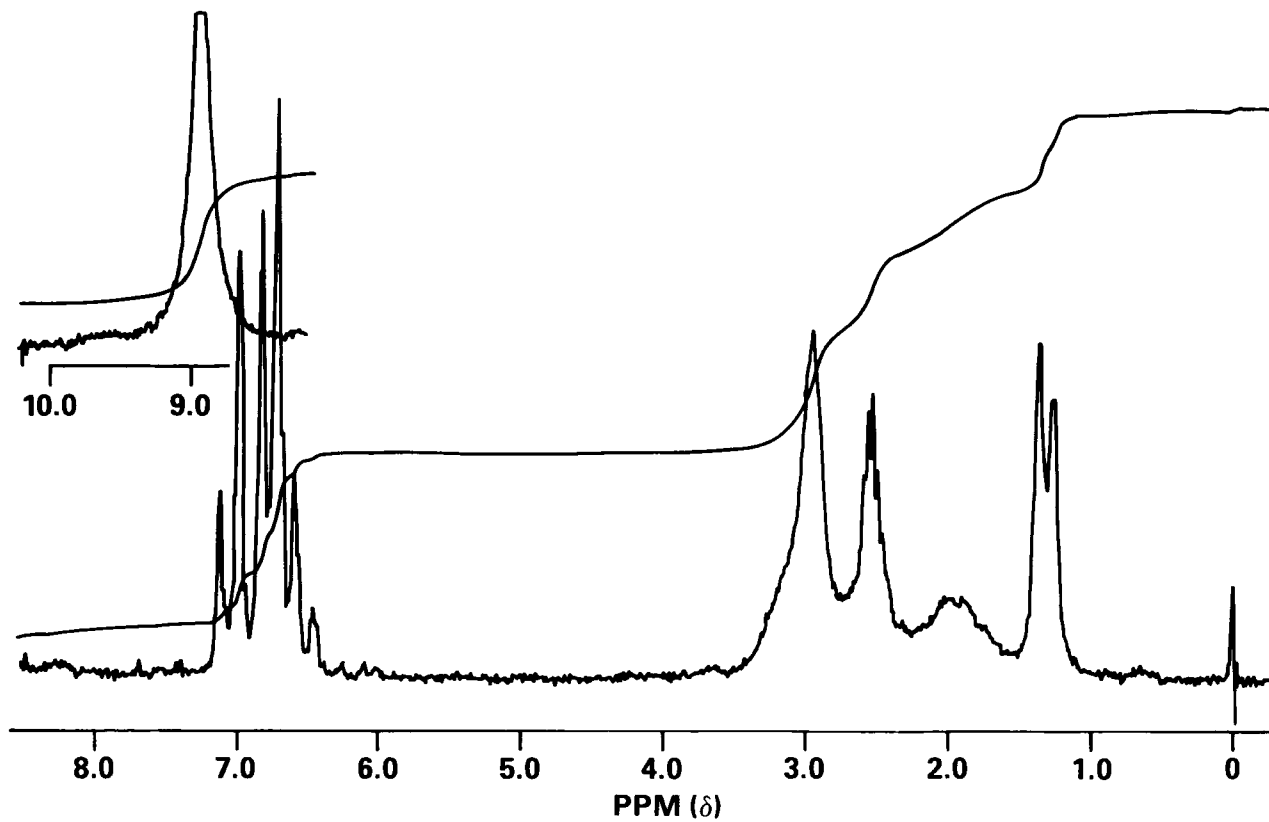


Figure 4. Nuclear Magnetic Resonance Spectrum of Dobutamine Hydrochloride

Chemical Shift (ppm)	Multiplicity	Area	Assignment
1.3	doublet	3	a
1.9	unresolved multiplet	2	b
2.5	unresolved (overlapping solvent)	2	c
3.0	unresolved triplets	5	d, e, f
6.8	overlapping multiplets	7	aromatic protons
8.9	very broad singlet	5	g, h, i, j

2.10 Mass Spectrum

The mass spectrum of dobutamine hydrochloride given in figure 5 shows the molecular ion of the free base at m/e 301. The major fragmentation consists of a cleavage beta to the nitrogen to yield peaks at m/e 123 and 178 with relative intensities, to the base peak, of 15.2 and 97.6%, respectively. The base peak is at m/e 107.

3. Synthesis

A mixture of crotonic acid, thionyl chloride, and a catalytic amount of dimethylformamide is stirred in a solvent such as benzene to give 2-butenoyl chloride (I). The Friedel-Crafts reaction of methoxybenzene (II) with (I) using $AlCl_3$ in carbon disulfide yields 1-(4-methoxyphenyl)-2-buten-1-one (III). 3,4-Dimethoxybenzeneethanamine (IV) is then condensed with (III) to give 3-[[2-(3,4-dimethoxyphenyl)ethyl]amino]-1-(4-methoxyphenyl)-1-butanone (V). This ketone is reduced with hydrogen over Pd/C to give N-[2-(3,4-dimethoxyphenyl)ethyl]-4-methoxy- α -methylbenzene-propanamine (VI). An alternate synthesis for compound (VI) involves the reduction of 4-(4-methoxyphenyl)-3-buten-2-one (VII) with hydrogen over Raney nickel to yield the corresponding butanone (VIII), which is then condensed with (IV) to produce the imine (IX). Compound (IX) is then reduced again with hydrogen over Pd/C to give (VI).

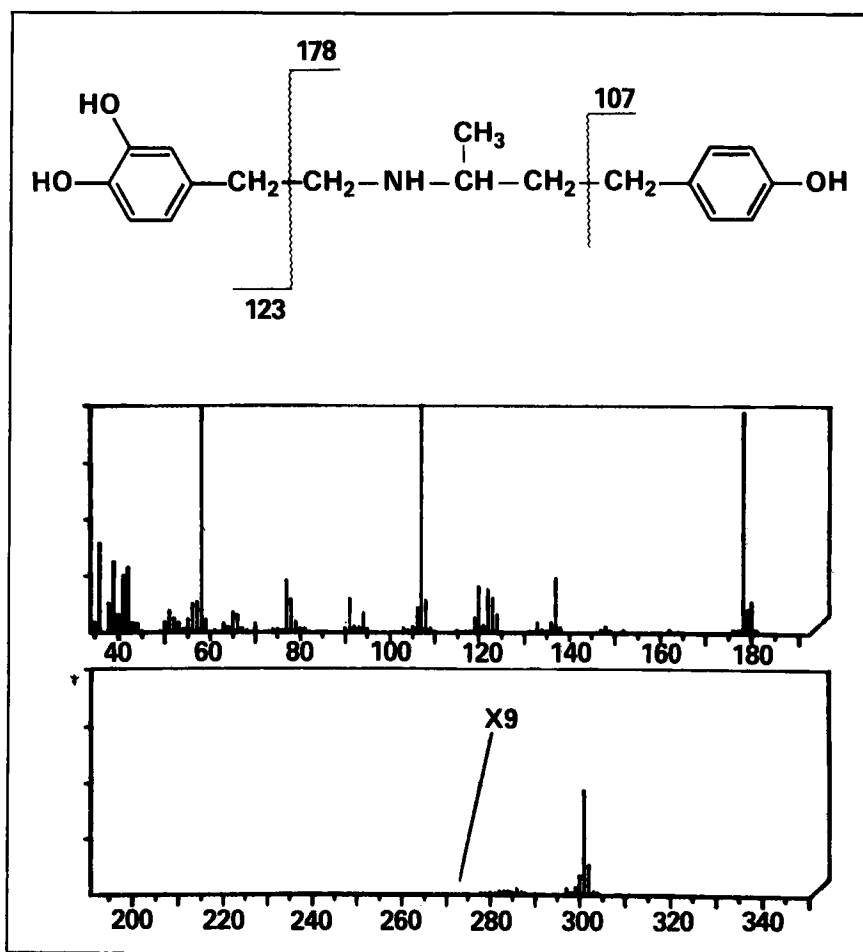


Figure 5. Mass Spectrum of Dobutamine Hydrochloride

A second alternate synthesis of compound (VI) involves the reaction of 4-methoxy- α -methylbenzene-propanamine (X) with 3,4-dimethoxybenzeneacetic acid (XI) at 200°C to yield 3,4-dimethoxy-N-[3-(4-methoxyphenyl)-1-methylpropyl]benzeneacetamide (XII), which is then reduced with borane in THF to produce (VI). The trimethoxy secondary amine (VI) is demethylated by refluxing its solution in glacial acetic acid and HBr to yield dobutamine hydrobromide (XIII). Compound (XIII) is added to aqueous methanol then small amounts of hydrochloric acid are added to produce dobutamine hydrochloride (XIV). The flow diagram of the synthesis presented above (2) is shown in figure 6.

4. Stability-Degradation

Dobutamine hydrochloride is quite stable to refluxing in acid and to heating in air for 20 hours at 115°C (2). However, the drug is very rapidly oxidized to the corresponding aminochrome at pH 11-13. Approximate kinetic measurements suggest a half life of 30-45 minutes. This is similar to catecholamines which produce aminochromes that undergo further rapid and complex oxidations and/or condensations. These reactions yield products of unknown structure which finally are converted to dark colored polymers related to the melanins. Photolysis of an aqueous solution of the drug at 40-50°C for 5 days using a 325 w mercury lamp in the presence of oxygen also produced the aminochrome as the photooxidation product.

5. Absorption, Metabolism, and Excretion

5.1 In Dog

The short plasma half-life of dobutamine (1-2 minutes) was found by Murphy et al. (4) to be due to the rapid redistribution of the drug from the plasma to the tissue. However, plasma half-life of radioactivity following the administration of ¹⁴C-dobutamine was 1.9 hours. The major circulating metabolite is the glucuronide conjugate of 3-O-methyldobutamine. During a continuous intravenous infusion of dobutamine, the plasma level of the parent drug reach a maximum within 8 to 10 minutes, while those of the metabolites peak be-

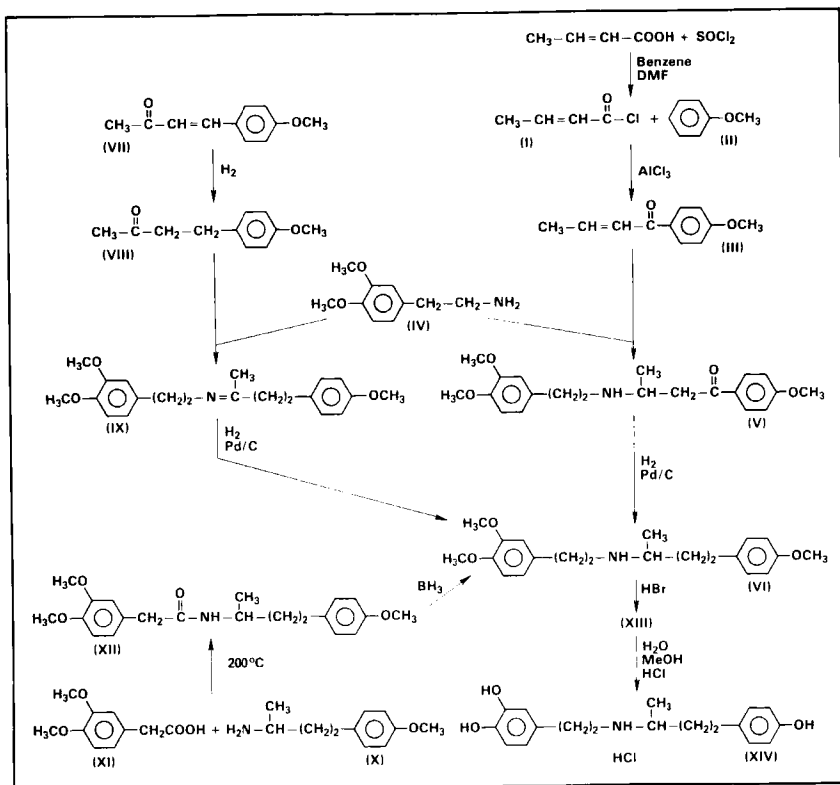


Figure 6. Synthesis of Dobutamine Hydrochloride

tween 3 and 4 hours. Dobutamine and/or its metabolites are eliminated via the urine and bile in both the dog and rat (5). After 48 hours from administering ^{14}C -dobutamine to dogs, 67% of the radioactivity was excreted in urine and 20% in feces. Dogs with cannulated bile ducts excreted 30 to 35% of the administered drug in the bile. The major urinary metabolites are the glucuronide conjugates of both dobutamine and 3-O-methyldobutamine. At very high doses of the drug, small amounts of hydroxylated dobutamine and hydroxylated 3-O-methyldobutamine were observed in the urine. The exact position of the extra hydroxyl group was not determined. The metabolites were not observed at therapeutic dosages.

5.2 In Man

Serum levels of dobutamine reached a maximum of 20 ng/ml during a 15-minute infusion of dobutamine at a rate of $2\text{ }\mu\text{g/Kg/min.}$ and declined to 3 ng/ml within 5 minutes after the infusion. Rapid clearance from the plasma is indicated by its short half-life of approximately 2 minutes. Dobutamine is rapidly metabolized by methylation and conjugation to 3-O-methyldobutamine and conjugates of dobutamine. The major portion of the metabolites are excreted in the urine within the first 2 hours following infusion and the remainder within 6 hours. Metabolism and excretion in man are similar to the processes described above in the dog. For the detailed pharmacological and biochemical properties of dobutamine, the reader should consult the profile by Weber and Tuttle (5).

6. Methods of Analysis

6.1 Elemental Analysis (As $\text{C}_{18}\text{H}_{23}\text{NO}_3\cdot\text{HCl}$)

<u>Element</u>	<u>Theory (%)</u>
C	63.99
H	7.16
N	4.15
O	14.21
Cl	10.49

6.2 Chloride Identity

About 5 ml of dobutamine hydrochloride solution in water, 1 mg/ml, is acidified with two drops of concentrated nitric acid. When a drop of 0.1 N silver nitrate is added to this solution a white precipitate is formed which is readily solubilized by the addition of 3 drops of ammonium hydroxide. This indicates the presence of chloride ion.

6.3 Non-Aqueous Titration

The secondary amine function of dobutamine hydrochloride may be determined by potentiometric titration with perchloric acid using glacial acetic acid as a nonaqueous solvent. Mercuric acetate is used to tie up the chloride ion.

6.4 Chloride Determination

A sample of dobutamine hydrochloride containing at least 2 mg of chlorine is ignited in a Schöniger flask containing 20 ml of water. Three drops of diphenyl carbazone (5 mg/ml) in methanol are added to the solution of the completely burned sample. Mercuric nitrate, 0.5 N, is then used to titrate this solution to the first sign of rose color, using a 1 ml microburette:

$$\text{percent chlorine} = \frac{\text{ml mercuric nitrate} \times \text{normality} \times 35.5 \times 100}{\text{mg sample}}$$

$$\text{percent purity of dobutamine hydrochloride} = \frac{\text{percent chlorine found} \times 100}{\text{percent chlorine theory}}$$

6.5 Chromatography

6.5.1 Thin Layer Chromatography

The R_f value for dobutamine hydrochloride when chromatographed on a silica gel 60 F254 thin layer plate developed by ethyl acetate/n-propanol/water/acetic acid (100/40/15/5 v/v/v/v) in an unsaturated chamber is about 0.67. The spot of the drug may be visualized under short wavelength UV light (254 nm), or under white light after exposure to iodine vapors.

6.5.2 Gas Chromatography

Silylated dobutamine hydrochloride (by reaction with N-trimethylsilylimidazole) may be chromatographed on a 3 foot glass column packed with 3% OV-225 on Chromosorb G AW-DMCS (100/120 mesh). The column is operated at 230°C using helium as a carrier gas at the rate of 60 ml/min. The retention time of the drug is approximately 4 minutes. A flame ionization detector is used. n-Triacontane is used as an internal standard.

6.5.3 High Performance Liquid Chromatography

Dobutamine hydrochloride may be analyzed on a C₁₈ reversed-phase column eluted with 75% 0.05M KH₂PO₄, pH 4.4, and 25% methanol at 2 ml/minute. The compound is detected at 280 nm. The retention time of the drug is approximately 6 minutes.

7. Analysis of Biological Samples

7.1 Enzymatic Assay

Plasma levels of dobutamine hydrochloride are determined by reaction of the drug with 3H-methyl-S-adenosylmethionine in the presence of catechol O-methyl-transferase. The radioactivity of the labeled methyl derivative is determined by a liquid scintillation counter using an external standard. The final recovery of added dobutamine as 3H-CH₃-dobutamine is 24.9 ± 1.3% in the range of 2 to 170 ng/ml (4). When ¹⁴C-dobutamine is administered the samples are counted by a double isotope method.

7.2 Chromatographic Assays

7.2.1 Thin Layer Chromatography

A Silica gel G plate is developed with a 15% aqueous solution of NaHSO₃. The R_f values in this system for dobutamine and 3-O-methyldobutamine are 0.50 and 0.35, respectively (4).

7.2.2 Gas Chromatography

Plasma and urine levels of the drug are determined by chromatographing the trimethylsilyl derivative of dobutamine on a 6-foot column packed with 3.0% UC-W98 silicon gum rubber (methylvinyl) on Diatoport S operated at 260°C. The hydrogen flame detector is maintained at 280°C. Helium flow rate is 60 ml/min. The retention time of dobutamine derivative (TMS) under these conditions is 3.8 minutes. This method measures plasma levels as low as 1 µg/ml (4).

The levels of free and conjugated 3-O-methyldobutamine in plasma and urine are determined using electron capture detection of the pentafluoropropionate derivative of the metabolite. A 4-foot coiled column is packed with SP-2100 and maintained at 240°C. The temperature of the ⁶³Ni electron capture detector is 250°C. The retention time of the pentafluoropropionate derivative is 1.6 minutes. Plasma levels as low as 50 ng/ml are readily measured using this method. 3-Hydroxy-N-3-(4-hydroxyphenyl)-1-methyl-N-propylphenethylamine hydrobromide is used as an internal standard.

7.2.3. High Performance Liquid Chromatography

Dobutamine hydrochloride may be determined in plasma levels, after extraction, on a C₁₈ reversed-phase column eluted with 22% acetonitrile-78% 0.1 M phosphate buffer (pH 2.0) at 2 ml/minute. The drug and its metabolite are detected by a fluorescent detector with an excitation wavelength of 195 nm and a 330 nm emission cut off filter. The retention times of dobutamine and the 3-methoxy metabolite are 5.2 and 7.9 min., respectively. The lower limit of sensitivity is 10 ng/ml. Reproducibility is ± 5% over a 25-300 ng/ml range. Nylidrin is used as an internal standard (6).

7.3 Mass Spectrometry (GC/MS)

The biological samples are analyzed with an LKB 900 GC mass spectrometer containing a 4-foot coiled glass column packed with 1% UC-W98 on Gas Chrom Q. The column is maintained at 240°C and the

flow rate of helium is 60 ml/min. The ion source voltage is 70 eV. The retention times of the trimethylsilyl dobutamine and trimethylsilyl-3-O-methyldobutamine are 3.8 and 3.6 min., respectively. The mass spectrum of the dobutamine-TMS shows a molecular ion at 517 and fragments at 250 and 267. The spectrum of the derivatized metabolite has a molecular ion at 459 with major fragments at 250 and 209. This fragmentation pattern confirms the presence of the methyl group on the catechol moiety.

8. Analysis of Pharmaceutical Formulations

8.1 Chromatographic Assays

8.1.1 Thin Layer Chromatography

Samples are dissolved in methanol. The insoluble excipients are removed by centrifugation. The solution is applied on a silica gel plate using the same conditions as listed previously in section 6.5.1. Additional detection sensitivity may be obtained by spraying with a 6% solution of ferric chloride followed by a 2% solution of potassium ferricyanide.

8.1.2 Gas Chromatography

Samples are extracted into ethyl acetate from pH 9.0 buffer. After evaporation of the solvent, n-triacontane, the internal standard, in pyridine/chloroform is added to the residue. The trimethylsilyl derivative is formed and chromatographed according to the details mentioned in section 6.5.2.

8.1.3 High Performance Liquid Chromatography

Samples are dissolved in water to a concentration of approximately 0.5 mg/ml and injected directly into the liquid chromatograph without additional preparation. The conditions of section 6.5.3 apply also to the analysis of formulations.

8.2 Spectrophotometric (UV)

Dobutamine hydrochloride may be determined spectrophotometrically in 0.5 M hydrochloric acid at the maximum of 278 nm. If excipients interfere, the drug may be extracted into ethyl acetate from pH 9 buffer followed by extraction into 0.5 M hydrochloric acid for the UV measurement.

9. Acknowledgments

The authors are thankful to Dr. A. Hunt, Mr. H. W. Smith and Dr. A. D. Kossoy for their help in generating and permission to use the UV, crystallinity and stability degradation data.

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The literature is surveyed through January, 1979.

ERYTHROMYCIN

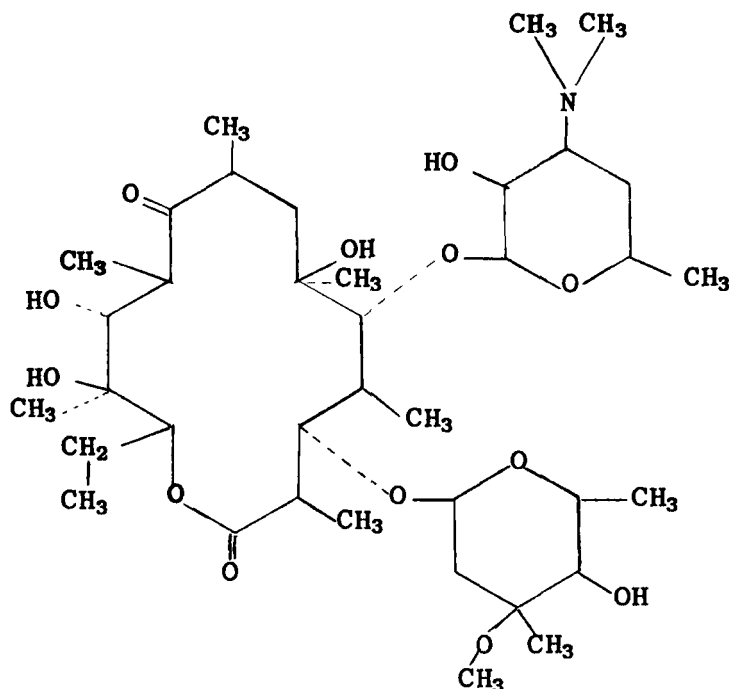
William L. Koch

1. Description
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1. Description

1.1 Name, Formula, Structure, and Molecular Weight

Erythromycin, erythromycin A



Erythromycin A

$C_{37}H_{67}NO_{13}$

Mol. wt.: 733.92

Erythromycin is a macrolide antibiotic consisting of the aglycone, erythronolide A; the aminosugar, desosamine; and the neutral sugar, cladinose.

Using NMR and CD studies,^{1,2} the conformation features were deduced (Fig. 1).

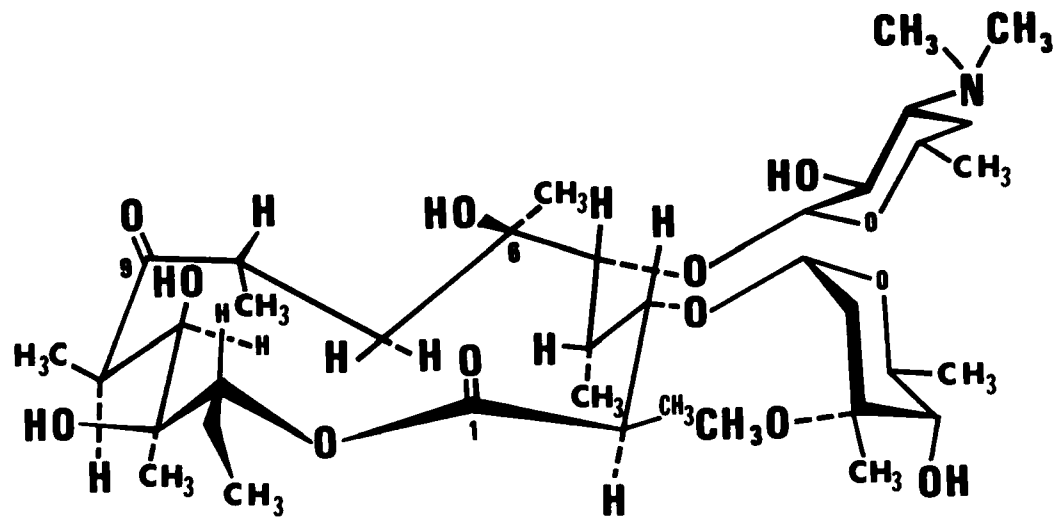


Fig. 1. Spatial arrangement of erythromycin

1.2 Appearance, Color, and Odor

The compound is a white crystalline powder, practically odorless, and has a bitter taste.

2. Physical Properties

2.1 Solubilities

Weiss *et al.*³ reported the solubility of erythromycin summarized in Table 1.

2.2 Infrared Spectrum

The infrared spectrum of erythromycin is commonly used for its identification. Figure 2 shows the spectrum of a 75 mg./ml. chloroform solution. The bands at 1685 and 1730 cm^{-1} are due to the ketone carbonyl and the lactone carbonyl, respectively. The absorption peaks between 1000 and 1200 cm^{-1} are due to the ethers and amine functions. The CH_2 bending is evidenced by peaks between 1340 and 1460 cm^{-1} , and alkane stretching peaks appear between 2780 and 3020 cm^{-1} . Hydrogen bonded OH and water appear as bands between 3400 and 3700 cm^{-1} .

2.3 Ultraviolet Spectrum

The ultraviolet spectrum of erythromycin in methanol exhibits λ_{max} at 288 nm. The ϵ value of 31.1 and the λ_{max} are consistent with the $n \longrightarrow \pi^*$ transition of $C = 0$.⁴

TABLE 1

Solubilities of Erythromycin

<u>Solvent</u>	<u>mg./ml.</u>
Isooctane	0.477
Petroleum ether	4.69
Cyclohexane	0.2*
Carbon disulfide	5.05
Carbon tetrachloride	> 20
Toluene	> 20
Benzene	> 20
Diethyl ether	> 20
Chloroform	> 20
Ethylene chloride	> 20
Methyl ethyl ketone	> 20
Acetone	> 20
1,4-Dioxane	> 20
Isoamyl acetate	> 20
Ethyl acetate	> 20
Isoamyl alcohol	9.65
Pyridine	> 20
Formamide	> 20
Benzyl alcohol	> 20
Isopropanol	> 20
Ethanol	> 20
Methanol	> 20
Ethylene glycol	> 20
Water	2.1

* Weiss reported this value as > 20. We found 0.2 in our laboratories.

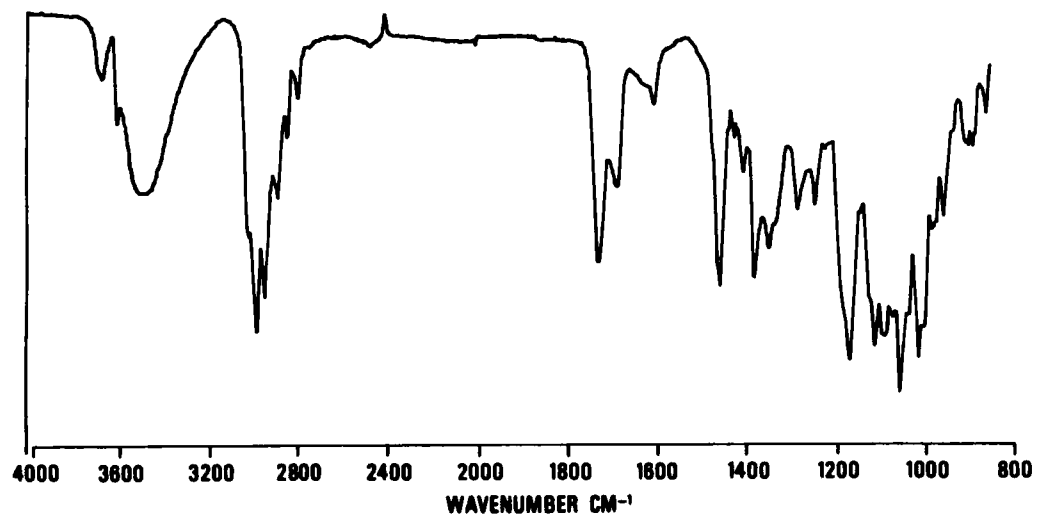


Fig. 2. Infrared absorption spectrum of erythromycin

2.4 Thermal Gavimetric Analysis

A TGA of erythromycin hydrate indicates a loss of volatiles of about 4%, then decomposition starting at 195°C.⁵

2.5 Differential Thermal Analysis

A DTA of erythromycin hydrate shows an endotherm at about 128°C., indicating simultaneous loss of volatiles and melting. A DTA of erythromycin anhydrate indicates melting starting at 193°C., then decomposition.⁶

2.6 X-Ray Diffraction

The x-ray powder diffraction patterns of erythromycin dihydrate and anhydrate are shown in Table II. Radiation: Cr/V, λ 2.2896.⁷

2.7 Nuclear Magnetic Resonance

The spectrum shown in Figure 3 was obtained as a CD₃OD solution using a Varian T-60A 60MHz instrument. General band assignments are listed in Table III according to Underbrink.⁸

2.8 pKa

The pKa for erythromycin in 66% DMF/34% water is 8.6.

TABLE II
X-Ray Powder Diffraction Data

Erythromycin			
Anhydrate		Dihydrate	
dÅ	I/I₁	dÅ	I/I₁
19.02	50	11.84	10
15.63	10	9.01	50
13.40	70	8.57	100
11.14	60	7.26	10
10.11	60	6.73	100
9.74	80	6.40	20
8.43	100	6.12	40
7.79	60	5.43	30
7.24	70	5.05	40
6.79	15	4.99	30
6.43	60	4.60	30
6.31	60	4.42	20
6.02	15	4.25	20
5.71	70	4.12	20
5.46	20	4.00	10
5.20	70	3.90	10
5.01	10	3.78	10
4.85	60	3.39	10
4.78	50	3.30	10
4.57	20	3.16	10
4.51	30	2.99	10
4.43	40	2.90	10
3.91	20		
3.75	10		
3.58	05		
3.35	02		
2.98	02		
2.23	02		
2.06	02		

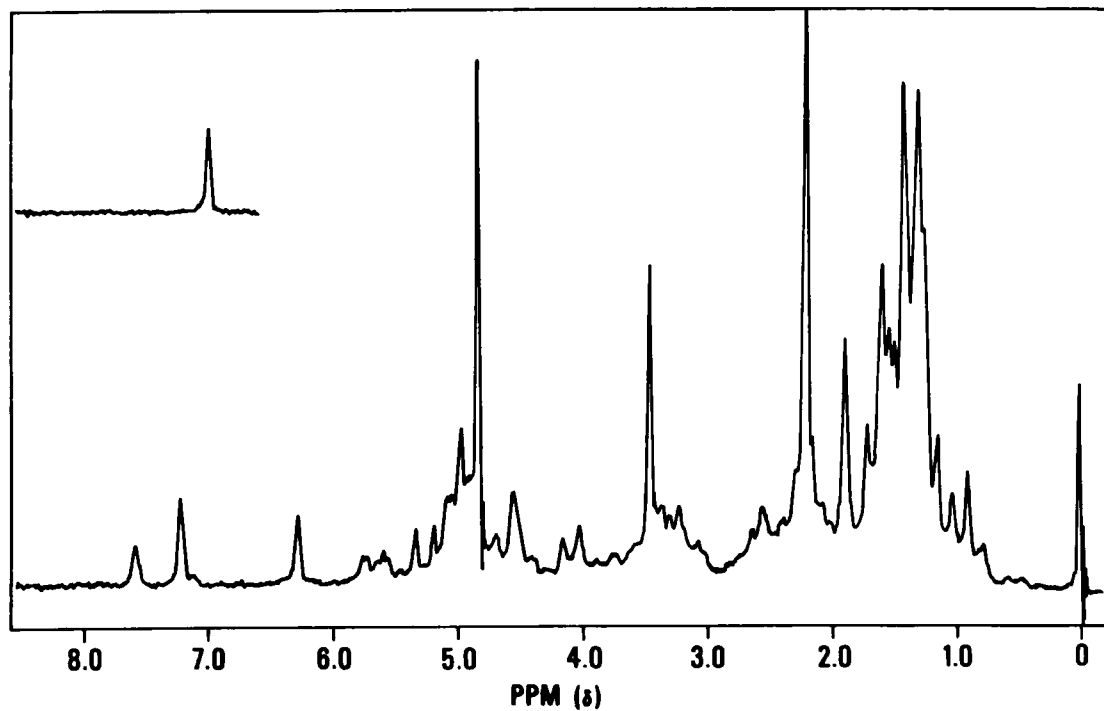


Fig. 3. NMR spectrum of erythromycin

TABLE III

NMR Spectral Assignments of Erythromycin

Solvent: CD_3OD

Instrument: Varian T-60A 60 mHz

<u>Proton(s)</u>	<u>Resonance Position (PPM)</u>	<u>Peak Type</u>
$\text{CH}_3\text{CH}_2 -$.9	Unresolved triplet
All methyls not listed above or below	1 -1.5	Overlapping singlets and doublets
$\text{N}(\text{CH}_3)_2$	2.35	Singlet
$-\text{OCH}_3$	3.35	Singlet (over- lapping solvent multiplet)

Other protons (methyne and methylene) give unresolved and overlapping multiplets in the 60 mHz spectrum of the CD_3OD solution.

3. Methods of Analysis

3.1 Ultraviolet Assay

The ultraviolet chemical assay for erythromycin remains largely unchanged from that described by Kuzel et al.⁹ in 1954. This procedure is essentially as follows. The reference standard, alkali reagent, and buffer solutions are prepared prior to the assay.

Phosphate buffer pH 7.0 is prepared by dissolving 13.6 g. KH_2PO_4 (anhydrous) and 27.2 g. K_2HPO_4 (anhydrous) in sufficient purified water to make 5 liters.

The reference standard solution is prepared by dissolving about 35 mg. accurately weighed erythromycin standard in 100 ml. methanol in a 250 ml. volumetric flask. This is diluted with phosphate buffer pH 7.0 to 250 ml., mixed, and allowed to cool to room temperature, then again diluted to the mark and mixed well.

The alkali reagent is prepared by slurring 42 g. $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ in about 125 ml. 0.5N NaOH in a 250 ml. volumetric flask. An additional 100 ml. purified water is added and the slurry heated on the steam bath to aid in solution. The solution is cooled slowly to room temperature and diluted to 250 ml. with purified water, then filtered prior to use.

Four 10 ml. aliquots of the standard solution are pipetted into separate 25 ml. volumetric flasks, two are labelled standard and the others blank. One ml. of 0.5N H_2SO_4 is added to the blank flasks and they are allowed to stand after mixing at room temperature for 60 minutes \pm 5 minutes. Two ml. of purified water are added to the standard flasks. At the end of this time, 1.0 ml. of 0.1N NaOH is added to the blank flasks and their contents swirled

to mix. Then, 2.0 ml. of alkali reagents are added to all four flasks, they are swirled to mix and placed in a 60°C. water bath for 15.0 minutes. The flasks are then cooled rapidly in an ice bath, brought to room temperature, then diluted to 25.0 ml. with purified water. The UV absorbance is read at 236 nm. versus purified water in 1.0 cm. silica cells. The blank values are subtracted from the standard values and the average net absorbance used for calculation.

Bulk erythromycin raw material is treated the same as the standard. Formulations are made up to the same concentration as the standard in methanol and buffer and 10 ml. aliquots used for chromophore development.

The sulfuric acid treated aliquot representing the blank forms a cyclic ether anhydroerythromycin.¹⁰ The alkaline treatment causes the formation of an α,β unsaturated ketone (9-keto-10-ene) having its absorbance maximum as a shoulder at 236 nm. (ϵ 6000).^{11,12} Thus, any other UV absorbing species are measured with the blank and subtracted from the absorbance before calculation of the erythromycin concentration. A typical spectrum is shown in Figure 4.

3.2 Gas Chromatographic Assay

Tsuji and Robertson¹³ reported a gas chromatographic procedure for erythromycin using an OV-225 column or a PPE-20 column. The procedure involves silylating 10 mg. erythromycin with a mixture of trimethylchlorosilane, N,O-bis-trimethylsilylacetamide, and N-trimethylsilylimidazole in pyridine for 24 hours at 75°C. Ten micrograms are injected onto the column (3 mm. x 1850 mm., 3% OV-225 on GCQ100-120 mesh or 3 mm. x 1850 mm. 3% PPE-20 on Supelcoport at 275°C.) of an F and M model 400 gas chromatograph equipped with a flame ionization detector. They reported being able to separate erythromycins A, B, C,

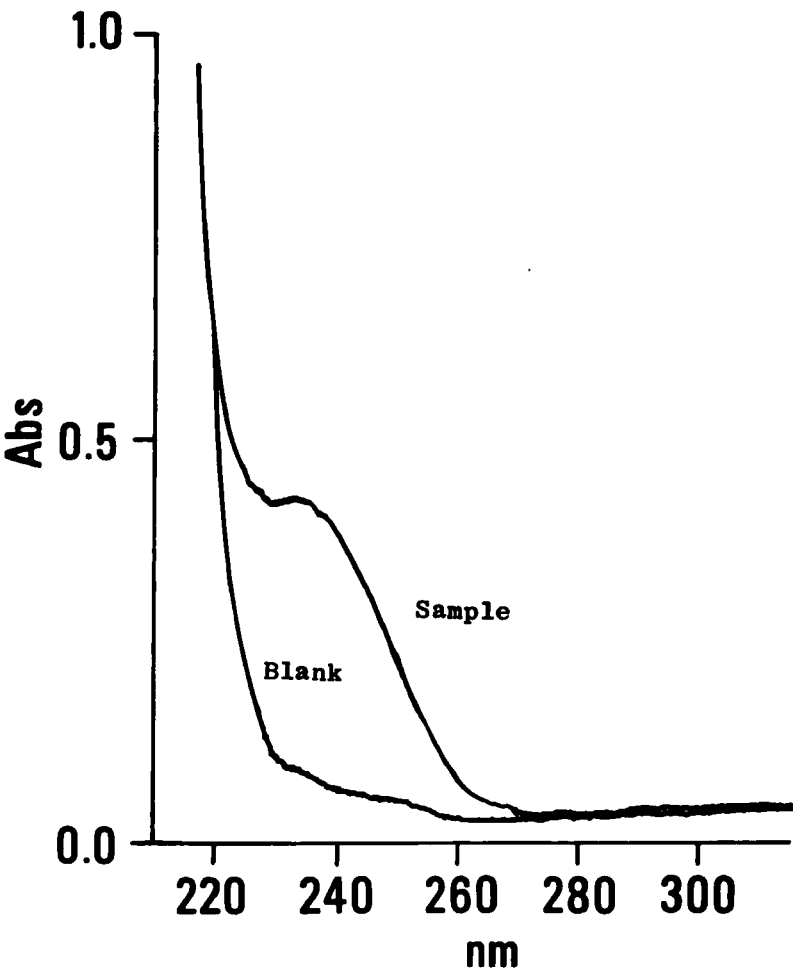


Fig. 4. UV spectra of sample and blank after chromophore development

anhydroerythromycin A, and erythralosamine. Good agreement with the microbiological assay is shown. However, the biggest drawbacks appear to be in silylation time and the instability of the GC column, 3 weeks at 275°C.

These authors¹⁴ later report using the GC method for enteric coated tablets of erythromycin, giving a recovery of 99.8% and a coefficient of variation of 2.3% based on placebo tablets spiked with erythromycin.

3.3 Colorimetric Assays

Two procedures are worthy of note here. The first, published in 1967 by Kuzel and Coffey¹⁵ is based on the ion pair dye complex of bromocresol purple (5',5"-dibromo-o-cresol-sulfonphthalein) and the desosamine moiety of erythromycin in pH 1.2 buffer. The method lacks specificity for erythromycin, measuring all tertiary amines; however, it is quite sensitive and precise, being routinely used for concentrations of 250 mcg. erythromycin/ml. in tablets and 20-100 mcg./ml. in fermentation broth. A more recent method by Sanghavi and Chandramohan¹⁶ is also based on a complex of the desosamine moiety, but they use p-dimethyl amino benzaldehyde as the coupling agent. The procedure is non-specific, but sensitive and linear over a concentration range of 10-35 mcg./ml.

3.4 Thin Layer Chromatography

Egon Stahl¹⁷ described four TLC systems. Table IV summarizes the solvents and R_f 's on silica gel G.

TABLE IV

<u>Solvent</u>	<u>R_f</u>	<u>Detection</u>
Methanol	.16	Brownish-green color after spraying with 10% sulfuric acid and heating 5-10 minutes at 80°C.
Chloroform-methanol 95 + 5	.03	
Chloroform-methanol 50 + 50	.29	
Butanol-acetic acid-water 60 + 20 + 20	.39	

Spraying with 10% molybdophosphoric acid in alcohol, followed by heating produces a blue spot on a yellow background. The spot disappears in 2 hours.

Vilim et al.¹⁸ devised a TLC identification system for erythromycin base, stearate, estolate and ethylsuccinate. This combination cannot differentiate between the estolate and ethylsuccinate.

Our laboratories¹⁹ have developed a system separating erythromycin estolate, erythromycin base, and anhydroerythromycin on a silica gel 60 F-254 plate utilizing ethanol, methanol, triethylamine, 170:30:1. Visualization is made by spraying with 0.15% xanthidol and 7.5% acetic acid in water. Table V summarizes the R_f's.

TABLE V

<u>Component</u>	<u>R_f</u>	<u>Color</u>
Erythromycin Base	0.30	Violet
Anhydroerythromycin	0.43	Violet
Erythromycin Estolate	0.60	Violet

3.5 Microbiological Analysis

Kavanagh and Dennen²⁰ report microbiological turbidimetric and plate assays for erythromycin base in Analytical Microbiology, Vol. 1. Staphylococcus aureus (ATCC 9144) is used for the turbidimetric procedure. The bulk raw material official assay is found in 21CFR - 452.10 and the official tablet assay is found in 21CFR452.110. The sample is diluted from 0.3 to 2.0 $\mu\text{g./ml.}$ in pH 7.0 buffer and comparison is made to a standard curve of 0, 0.3, 0.4, 0.6, 0.8, 1.2, 1.6, and 2.0 $\mu\text{g./ml.}$ Sarcina lutea (ATCC 9341) is used for the plate assay. A linear response in the range of 0.5 - 2.0 $\mu\text{g./ml.}$ is obtained when pH 8.0 buffer is used for sample and standard. In both methods, a small amount of methanol is used to solubilize the erythromycin prior to buffering at pH 7.0 or 8.0.

3.6 High Performance Liquid Chromatography

Omura et al.²¹ used a reverse phase high performance liquid chromatographic column, JASCO PACK SV-02-500[®], for macrolide antibiotics with methanol, M/15 acetate buffer pH 4.9, and acetonitrile (35:60:5) as solvent. A variable wavelength UV detector using the absorption of the individual compounds gave the required sensitivity. Alterations of buffer pH and the composition ratio of the mobile phase gave selectivity for separation of individual macrolide antibiotics.

Z. H. Hash²² reported chromatographic conditions for separating anhydroerythromycin from erythromycin using a normal phase Corasil II[®] silica gel column, with chloroform as mobile phase and refractive index detection.

White et al.²³ devised a reverse phase high performance liquid chromatographic procedure for erythromycin. Refractive index detection was used since the compound absorbs weakly in the UV. A 10 μm C₁₈/Lichrosorb[™] reverse phase column was used with 80% methanol, 19.9% water, 0.1% ammonium hydrochloride as the developing solvent.

Tsuji and Goetz²⁴ developed a quantitative high performance liquid chromatographic method for separating and measuring erythromycins A, B, and C, their epimers and degradation products. This method uses a μ Bondapak[®] C₁₈ reverse column with acetonitrile-methanol-0.2M ammonium acetate-water (45:10:10:25) as solvent. The pH and composition of the mobile phase may be adjusted to optimize resolution and elution volume. The authors utilized the procedure on USP reference standard and report a relative standard deviation of $\pm 0.64\%$.

4. Stability

Erythromycin is unstable in acidic or alkaline solutions and shows its maximum stability between pH 6.0 and 9.5²⁵. Its aqueous, alcoholic solution buffered at pH 7.0 - 8.0 is stable for about one week under refrigeration.

5. Bioavailability

Maximum levels of erythromycin in serum are obtained in 1 to 2 hours after a single dose. The U. S. Dispensatory²⁶ reports maximum serum levels of 0.2 μ g./ml. 1 hour after administration of a 250 mg. dose, 0.6 μ g./ml. 2 hours after a 500 mg. dose, and 1.2 μ g./ml. 2 hours after a 1 g. dose. Higher blood levels are achieved on a multiple dosage schedule. Since it is acid labile, a resistant coating is used in tablet formulations to overcome the deleterious effect of gastric fluid on erythromycin base; or the stearate salt is prepared which does not dissolve readily in the stomach.

The Bioavailability Monograph for Erythromycin²⁷ provides data for comparison of several manufacturers' tablets of erythromycin. The criteria for bioavailability tests are discussed.

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GRAMICIDIN

Glenn A. Brewer

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1. Introduction

Gramicidin is a peptide antibiotic first isolated by Dubos in 1939¹ as a crude complex together with a second peptide antibiotic known as tyrocidin. The mixture of the two antibiotics was called tyrothricin. Although the mixture was discovered about ten years after penicillin², tyrothricin was the first antibiotic utilized in clinical practice.

It was soon found that tyrothricin causes severe hemolysis when administered parenterally and was destroyed when given orally. The antibiotic complex or individual components are effective topically and are used in various cream, ointment, lotion and solution preparations alone or in combination with other antibiotics or topical steroids³.

The aspect of gramicidin which is of most interest to the analytical chemist is the continued study of the structure of the antibiotic, from its discovery to the present day. As more and more sophisticated separation and structural elucidation techniques have been developed, various scientists have applied these to the problem of understanding the complete structure of the gramicidin complex. Thus, we can trace the development of our understanding of the structure from the crude extract prepared by Dubos¹ to our current knowledge of not only the structure of the various components of gramicidin, but the conformation of the major fraction in solution.⁴ It is indeed interesting that so many scientists have applied their knowledge and skill to solve this difficult structural problem, considering the relative minor role of this material product in modern medicine.

2. Chemistry

Tyrothricin was obtained by acidification of the fermentation broth of Bacillus brevis to precipitate the antibiotic activity along with various proteins and then dissolving the antibiotic complex in alcohol. The alcohol was removed under vacuum, the residue was washed with ether, then redissolved in alcohol and finally reprecipitated with 1% sodium chloride^{5,6}.

It was soon recognized that tyrothricin was not a pure compound but could be separated into a neutral fraction called gramicidin and a basic fraction called tyroci-

dine by extraction with acetone and ether mixtures. The individual fractions were thought to be homogeneous because they were crystalline and had constant physical properties on recrystallization^{7,8,9,10,11,12}. At the time, these constant properties were considered to be sufficient proof to indicate chemical purity.

An empirical formula of $C_{74}H_{106}N_{14}O_{14}$ was proposed for gramicidin based on elemental analysis and a Rast molecular weight determination⁸. The application of various biochemical methods led the investigators to the conclusion that gramicidin was a peptide containing ten α -amino acid residues and a saturated aliphatic fatty acid containing 14 to 16 carbon atoms. It was established that a hydrolysate of gramicidin contained tryptophane and that histidine, arginine, tyrosine and ammonia were absent. The authors further noted that about half of the amino acid residues have the D-(unnatural) configuration. This was shown by oxidation with d-amino acid oxidase^{8,12}.

Other workers began to study the structure of gramicidin. Christensen and coworkers¹² isolated crystalline tryptophane and leucine from a hydrolysate. They found no evidence for a fatty acid component and established that phenylalanine, proline and hydroxyproline were absent from a hydrolysate. These workers isolated alanine dioxypyridate from a hydrolysate and also established that gramicidin contained a compound with vicinal hydroxy and amino groups. They speculated that this compound might be serine or isoserine and proposed that gramicidin contains two tryptophane, 2 leucine, 2 or 3 alanine and 1 hydroxyamino residues or a multiple of this composition.

Hotchkiss¹⁴ isolated optically and analytically pure d-leucine from the hydrolysate. This was the first non-enzymatic proof that d-amino acids actually occurred in gramicidin. He also noted the presence of an amino-hydroxy compound, but indicated that it was not isoserine.

Gordon, Martin and Synge¹⁵ utilized their new and elegant technique, chromatography, to establish the amino acid composition of gramicidin. They proposed a 24 unit cyclic peptide consisting of six moles each of leucine, and tryptophane, 5 moles of valine, 3 moles of alanine and 2 moles each of glycine and unknown hydroxyamino compound. They confirmed that the leucine was the d-form while the tryptophane and alanine had the L-configuration. The

valine appeared to be racemic.

Christensen¹⁶ isolated the dipeptide valylvaline from completely hydrolyzed gramicidin. This worker later showed that he had isolated a racemic mixture of D(-)-valyl-D(-)-valine and L(+)-valyl-L(+)-valine rather than dipeptides containing one d and one L-residue.¹⁷

Synge¹⁸, using starch columns, confirmed the presence of valylvaline and identified L-valylglycine in partial hydrolysates. This unexpected finding triggered a good deal of work on the kinetics of peptide hydrolysis in an attempt to develop a rational explanation.

Synge²¹ isolated the elusive hydroxyamino compound by azeotropic distillation and identified it as 2-aminoethanol. He proposed a structure of 6 L-tryptophane, 6 D-leucine, 5 D and L-valine, 3 L-alanine, 2 glycine and 2 aminoethanol residues.

There was some evidence that the preparations of gramicidin used in this structural work were not completely homogeneous^{15,22} but the evidence was not strong until Gregory and Craig²³ separated crystalline gramicidin into three major fractions by counter current distribution. Fraction B contained only 55% as much tryptophane as Fraction A based on an ultraviolet analysis. The third fraction was called gramicidin C.

Still using heterogeneous gramicidin, Synge²⁴ isolated the D-leucylglycine, L-alanyl-D-valine and L-alanyl-D-leucine from partial hydrolysates of gramicidin. He also had less conclusive evidence for the tripeptides alanyl-valylleucine or alanylleucylvaline.

In a review paper, Dr. Synge²⁵ recapitulated the early structural work on gramicidin and indicated that x-ray diffraction was incapable of distinguishing a gramicidin fraction purified by counter current analysis from the heterogeneous starting material.

Hinman, Caron and Christensen²⁶ corrected the earlier report by Christensen¹⁶. They reported that the dipeptides found on the hydrolysis of gramicidin were D-valyl-L-valine and L-valyl-D-valine.

James and Synge²⁷ speculated on the nature of the non-peptide bonds in gramicidin.

Hodgkin²⁸ examined crystals of gramicidins A and B by x-ray diffraction. She estimated that the molecular weight of gramicidin A was approximately 3800, Previous estimates by chemical methods were approximately 7000. In 1953, Cowan and Hodgkin²⁹ published a second report on gramicidin B.

A provisional structure for gramicidin was proposed by Gavrillov and Akimova³⁰.

Okuda and coworkers³¹ determined the amino acid composition of gramicidins A, B and C. Ishii and Witkop³² established the complete optical assignment of the amino acids in gramicidin A using enzymatic degradation and quantitative gas chromatography. The composition established was 4 moles of L-tryptophane, 4 moles of D-leucine, 2 moles of D-valine, 2 moles of L-valine*, 2 moles of L-alanine, 1 mole of glycine and 1 mole of aminoethanol. (*The authors actually found 1.6 moles of L-valine and 0.6 moles of L-isoleucine. This indicated the possibility of the non-homogeneity of gramicidin A.)

The evidence that gramicidin A was actually heterogeneous was obtained by Ramachandran³³ using counter current distribution with more than 1000 transfers. The new gramicidin which contained isoleucine was called gramicidin D. This was an unfortunate choice of terminology since the crude mixture of gramicidin fractions had been previously called gramicidin D (gramicidin Dubos) to distinguish it from other gramicidins (S and J).

Sarges and Witkop^{34,35} established the amino acid sequence of gramicidins A and D. The same authors also established the amino acid sequences of gramicidins B³⁶ and C³⁷. In addition, they synthesized gramicidins A and D³⁸.

Gross and Witkop³⁹ subjected commercial gramicidin to counter current distribution. They found that gramicidins A, B and C all consisted of a pair of congeners containing primarily valine-gramicidins (80-95%) with some isoleucine-gramicidins (5-20%) as minor components. In addition, they isolated a more hydrophilic, strongly antibiotic group of antibiotics which they called gramicidin D.

Urry and coworkers^{40,41} proposed a left-handed helical structure for gramicidin A. This conformation can undergo ion induced relaxations which provides a mechanism for the movement of the ion along the channel. These workers confirmed this proposed structure by nuclear magnetic resonance spectrometry⁴².

Using circular dichroism and nuclear magnetic resonance spectrometry, Veatch and coworkers⁴ established that four conformational species of gramicidin A exist in solution. Two were postulated to be helices of opposite handedness.

3. Description

3.1 Composition, Formula, Molecular Weight

The gramicidin of commerce is a complex of at least four compounds. The identified fractions are called gramicidins A, B, C and D. The major component of the mixture is gramicidin A. (See Section 2.)

The mixture of gramicidins is called gramicidin [1405-97-6] or gramicidin D (Dubos) [1393-88-0]. The latter name is used to distinguish the gramicidin discovered by Dubos from gramicidins S and J.

As discussed in Section 2, the chemical structure of the various fractions has now been elucidated. The general formula for gramicidin is shown below, where R and R' are different amino acid residues depending on the particular type of gramicidin.

HCO-R-Glycine-L-Alanine-D-Leucine-L-Alanine-D-Valine-
L-Valine-D-Valine-L-Tryptophane-D-Leucine-R'-D-Leucine-
L-Tryptophane-D-Leucine-L-Tryptophane-NHCH₂CH₂OH

3.11 Gramicidin A [11029-61-1]

Gramicidin A consists of a pair of congeners containing primarily L-valine gramicidin A (80-95%) but also containing L-isoleucine gramicidin A (5-20%)^{39, 34}.

3.111 L-Valine Gramicidin A [4419-81-2]

R = L-valine

R' = L-tryptophane

 $C_{99}H_{140}N_{20}O_{17}$

Molecular Weight 1882.349

3.112 L-Isoleucine Gramicidin A [5536-03-8]

R = L-isoleucine

R' = L-tryptophane

 $C_{100}H_{142}N_{20}O_{17}$

Molecular Weight 1896.376

3.12 Gramicidin B [11041-38-6]

Gramicidin B consists of a pair of congeners containing primarily L-valine gramicidin B but also containing L-isoleucine gramicidin B³⁶.

3.121 L-Valine Gramicidin B [4422-52-0]

R = L-valine

R' = L-phenylalanine

 $C_{97}H_{139}N_{19}O_{17}$

Molecular Weight 1843.312

3.122 L-Isoleucine Gramicidin B

R = L-isoleucine

R' = L-phenylalanine

 $C_{98}H_{141}N_{19}O_{17}$

Molecular Weight 1857.339

3.13 Gramicidin C [9062-61-7]

Gramicidin C also consists of a pair of congeners containing primarily L-valine gramicidin C but also small amounts of L-isoleucine gramicidin C are present³⁷.

3.131 L-Valine Gramicidin C [58442-65-2]

R = L-valine

R' = L-tyrosine

 $C_{97}H_{139}N_{19}O_{18}$

Molecular weight 1859.312

3.132 L-Isoleucine Gramicidin C

R = L-isoleucine

R' = L-tyrosine

$C_{98}H_{141}N_{19}O_{18}$

Molecular Weight 1873.339

3.14 Gramicidin D [1405-97-6]

As previously discussed, the term gramicidin D has been used to designate the entire gramicidin complex of gramicidins A, B, C, D or the isoleucine component of gramicidin A³³. Gross and Witkop³⁹ have used it to name a minor and still undefined, polar fraction of the gramicidin complex.

In the rest of this Analytical Profile, the physical and chemical properties which will be described will be of the complex mixture of gramicidins A, B, C and D unless otherwise noted.

3.2 Appearance, Color, Odor

Gramicidin is described as a crystalline powder, which is white or nearly white and is odorless⁴³.

4. Physical Properties

4.1 Spectra

4.11 Infrared Spectra

The infrared spectrum of gramicidin has been reported by Hayden, et al.⁴⁴.

The infrared spectra of the U.S.P. gramicidin standard are presented in Figures 1 and 2⁴⁵.

4.12 Nuclear Magnetic Resonance Spectra

Nuclear Magnetic Resonance has been used to establish the conformation of the pure fractions of the gramicidin complex⁴².

The proton resonance spectrum of the U.S.P. gramicidin standard is shown in Figure 3⁴⁶.



FIGURE 1
Gramicidin U.S.P. Standard
Mineral Oil Mull

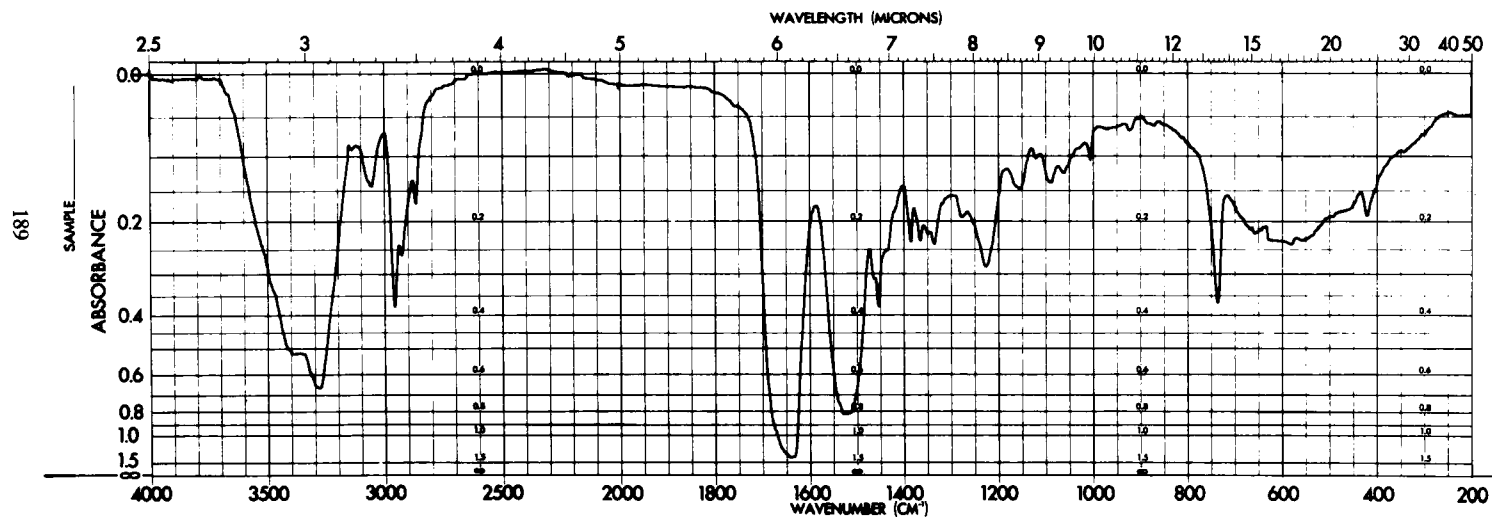


FIGURE 2
Gramicidin U.S.P. Standard
Potassium Bromide Pellet

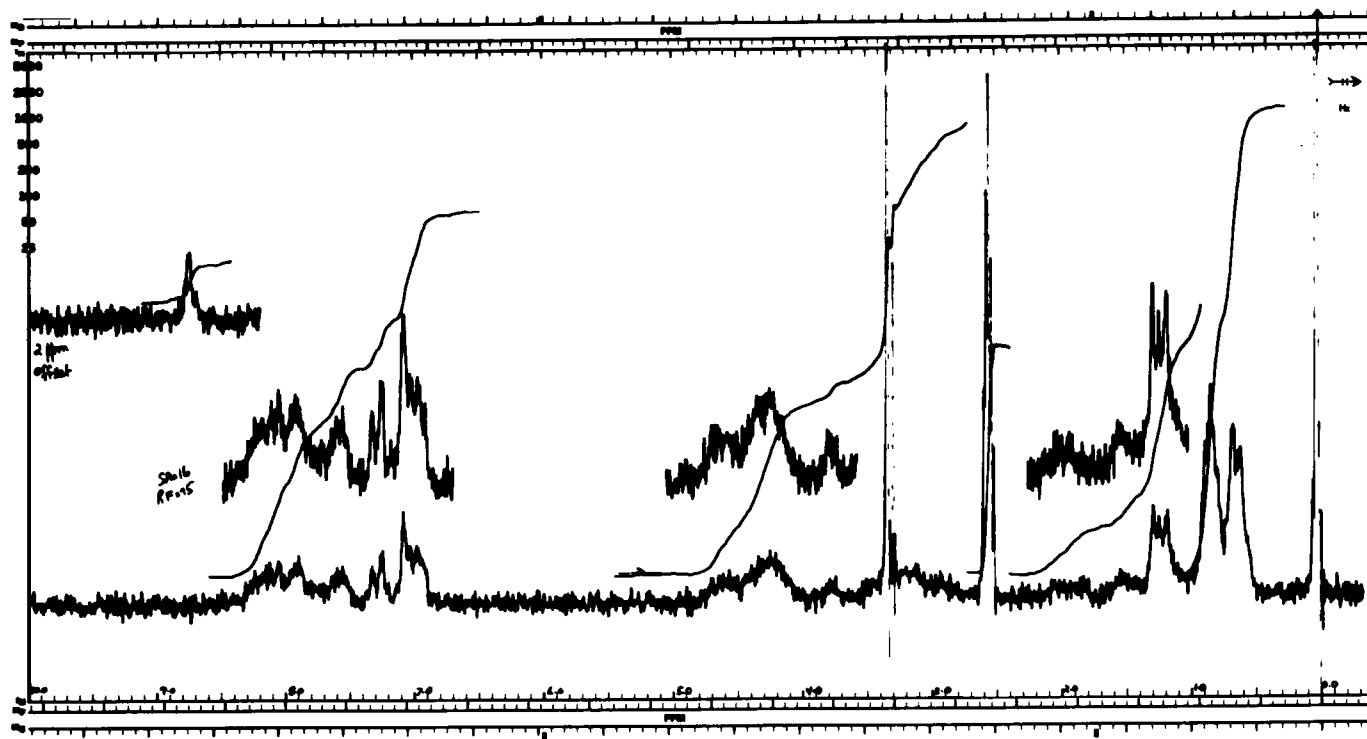


FIGURE 3
Gramicidin U.S.P. Standard
DMSO-d₆

4.13 Ultraviolet Absorption Spectra

The tryptophane content of the gramicidin complex was measured by the ultraviolet absorbance⁴⁷.

Cann has demonstrated the shift to longer wavelengths when acetic acid complexes with gramicidin⁴⁸.

The ultraviolet spectrum of the gramicidin complex has been reported by Hayden and coworkers⁴⁴.

Figure 4 presents the ultraviolet spectrum of the U.S.P. Reference standard taken in 95% ethanol⁴⁹.

4.14 Fluorescence Spectra

Sommermeier and coworkers⁵⁰ reported that solutions of gramicidin exhibit fluorescence when irradiated with soft x-rays.

Gramicidin exhibits strong fluorescence in 95% ethanol. The excitation maximum is at 286 nm and the emission maximum is at 337 nm. The fluorescence intensity was linear with respect to concentration in solution⁵¹.

4.15 Raman Spectra

Rothchild and Stanley studied the conformation of Gramicidin A using Raman spectroscopy⁵². Two types of conformation were found depending on the solvent used.

4.2 Crystal Properties

4.21 Crystalline Modifications

Dr. Synge noted that when gramicidin complex was crystallized from acetone, he obtained small crystals that were not suitable for x-ray diffraction²⁵. When the sample was allowed to crystallize from alcoholic solution by slow evaporation, large chunky crystals were obtained. These gave very good x-ray diffraction patterns.

Olesen and Szabo obtained crystals from ethanol and acetone⁵³. They found the crystals to have different solubility, melting point and x-ray diffraction patterns. Since acetone is retained in the crystalline lattice, it was indicated that the forms are pseudopolymorphs.

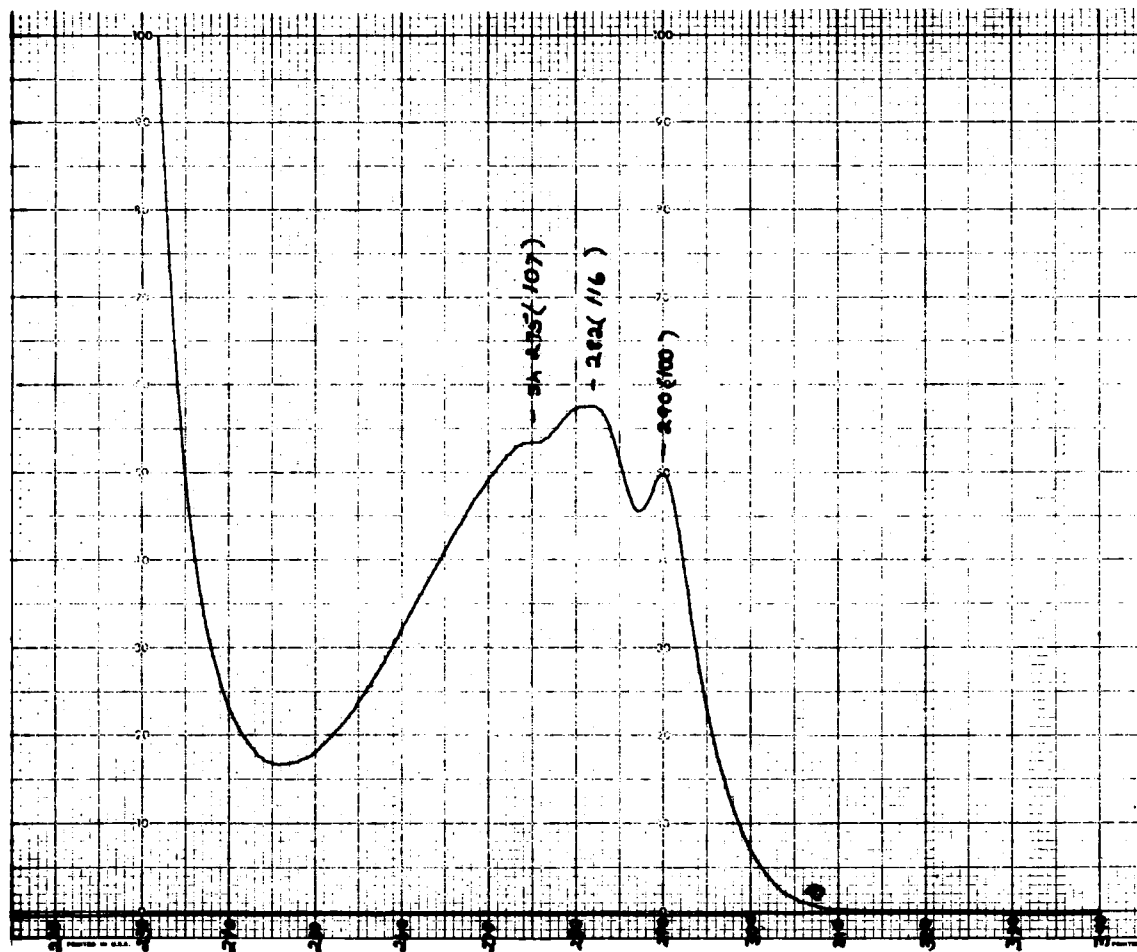


FIGURE 4
Gramicidin U.S.P. Standard
95% Ethanol

4.22 X-ray Powder Diffraction

As was mentioned in Section 2, x-ray diffraction was used in an effort to establish the structure of the gramicidin complex^{25,28,29}. These studies were frustrated by the fact that the gramicidin was not chemically pure but was a mixture of components.

Belavtseva found that crystalline gramicidin was converted to amorphous gramicidin by the action of x-rays⁵⁴.

The powder x-ray diffraction pattern of the gramicidin U.S.P. reference standard is shown in Figure 5⁵⁵. The relative peak intensities are presented in Table 1.

TABLE 1

Relative Peak Intensities of
U.S.P. Gramicidin Reference Standard as Measured
by Powder X-Ray Diffraction

<u>2θ</u> <u>(DEG.)</u>	<u>D</u> <u>(ANGSTROMS)</u>	<u>PEAK</u>	<u>REL. PEAK</u>	<u>AREA</u>	<u>REL. AREA</u>
6.64	13.31	143.5	1.000	850.0	1.000
7.40	11.95	104.1	0.725	791.4	0.931
9.44	9.37	28.4	0.198	301.2	0.354
13.69	6.47	31.1	0.217	327.6	0.385
14.46	6.13	28.6	0.199	246.0	0.289
16.75	5.29	46.5	0.324	349.9	0.412
16.92	5.24	45.3	0.316	274.6	0.323
17.86	4.97	48.7	0.339	473.6	0.557
19.39	4.58	63.8	0.445	716.2	0.843
20.07	4.42	50.7	0.353	414.9	0.488
21.26	4.18	38.8	0.270	288.3	0.339
21.68	4.10	39.5	0.275	250.9	0.295
22.28	3.99	37.2	0.259	275.6	0.324
23.55	3.78	36.2	0.252	312.1	0.367
23.89	3.72	34.6	0.241	306.0	0.360

4.23 Crystal Density

Low and Richards used a density gradient column to study the density of crystals of "mixed gramicidin fractions"⁵⁶.

4.24 Differential Thermal Analysis

The U.S.P. gramicidin standard shows a small endotherm at 161°C and a larger endotherm at 244°C. Both endotherms are broad⁵⁷.

4.3 Solubility

4.31 Solubility in Pure Solvents

The solubility of the gramicidin complex in a variety of solvents has been determined by Weiss and coworkers⁵⁸.

TABLE 2

Solubility of Gramicidin Complex
in Various Solvents

<u>SOLVENT</u>	<u>SOLUBILITY</u> <u>mg/ml</u>	<u>SOLVENT</u>	<u>SOLUBILITY</u> <u>mg/ml</u>
Water	0.140	Ethyl Acetate	11.90
Methanol	>20	Isoamyl Acetate	>20
Ethanol	>20	Methyl Ethyl Ketone	18.10
Isopropanol	>20	Acetone	18.80
Isoamyl Alcohol	14.10	Diethyl Ether	10.7
Cyclohexane	0.02	Ethylene Chloride	2.15
Benzene	0.19	1,4-Dioxane	>20
Toluene	0.04	Chloroform	>20
Petroleum Ether	0.007	Carbon Disulfide	0.100
Isooctane	0.005	Pyridine	>20
Carbon Tetrachloride	0.047	Formamide	>20
Ethylene Glycol	>20	Benzyl Alcohol	>20

4.32 Solubility in Solutions of Quaternary Ammonium Compounds

A number of reports have indicated that tyrothricin is more soluble in solutions of quaternary ammonium compounds^{59,60}.

When it was realized that tyrothricin was a mixture of antibiotics, the same principle was applied to gramicidin^{61,62,63,64,65}. The addition of quaternary pounds to gramicidin formulations to increase the solubility of the antibiotic in aqueous solution has been utilized^{66,67}.

4.33 Solubility in Solutions of Other Surface Active Agents

A number of other materials have been shown to increase the solubility of gramicidin in aqueous solution. Alcohols⁶⁸, xanthocillin⁶⁹, fatty acid amides⁷⁰, fatty acid alcohols⁷¹, aliphatic amines⁷² and polyvinylpyrrolidone^{73,74}.

4.4 Physical Properties of Solutions

4.41 Diffusion

The diffusion constants for the gramicidin complex were determined in acetic acid and ethanol solution⁷⁵. The molecular weight range of gramicidin was calculated as 2800-5000.

Polson using a similar method obtained a molecular weight of 3000⁷⁶.

4.42 Surface Tension

Gramicidin decreases the surface tension of aqueous solution⁷⁷. The bactericidal and hemolytic properties of gramicidin were destroyed by heat but the surface tension depression was not changed.

Kemp and coworkers found that when they partitioned gramicidin between water and heptane, it migrated to the walls of the vessel⁷⁸.

4.43 Specific Volume

Derechin and coworkers noted the anomalous behavior of gramicidin A in absolute ethanol⁷⁹. The apparent partial specific volume increased with decreasing concentration of gramicidin. The same was not true for aqueous-ethanol solutions.

4.44 Conformation

The conformation of gramicidin in aqueous solution has been extensively studied. A lipophilic left-handed helical structure has been proposed for gramicidin A^{40,41}. It was proposed that the mode of action of gramicidin is due to the formation of ion transport channels across biological membranes.

Bamberg and coworkers have studied the single channel conductance of gramicidins A, B and C^{80,81}. Significant differences between gramicidin A and B were found.

Cabon 13 NMR has been used to confirm the presence of a double helical dimer model⁸².

The conformation of gramicidin in various organic solvents has also been established^{4,83,84,85,86}.

Circular dichroism at high pressures has been used to study the conformation of a derivative of gramicidin A in trifluoroethanol solutions⁸⁷.

Kyogoku and Kawano have prepared an extensive review of the use of NMR techniques to study the conformation of gramicidin and other antibiotics in solution⁸⁸.

Lotz and coworkers have used poly (γ -benzyl-D-L-glutamate) as a stereochemical model to study the conformation of gramicidin A⁸⁹.

5. Production

5.1 Fermentation

The gramicidin complex was originally isolated by Dubos as a component of the antibiotic mixture called tyrothricin formed by an aerobic sporulating bacillus⁷.

Dubos and Hotchkiss found that a number of species of aerobic sporulating bacilli produced gramicidin¹⁰.

Stokes and Woodward established that Bacillus brevis produces the antibiotic in stationary cultures of both complex natural and synthetic media⁹⁰. They noted that production of the antibiotic occurred in an aerated synthetic medium but not in an aerated complex nitrogenous medium.

Konikova and coworkers compared the productivity of two strains of bacilli in the production of gramicidin⁹¹. Lewis and coworkers studied the production of the antibiotic complex by Bacillus brevis in both natural and complex media⁹². They noted requirements for calcium, magnesium and manganese ions. Stokes patented a submerged culture fermentation production procedure utilizing a synthetic medium⁶. Appleby and coworkers studied the addition of vitamins to synthetic medium⁹³. Konikova and Dobbert studied the effect of the addition of amino acids to a synthetic medium on the production of tyrothricin⁹⁴. They found that these additions had no effect on antibiotic production although growth was stimulated. Udalova and Fedorova have studied the effect of different carbon sources on the yield of antibiotic⁹⁵.

A number of workers have described the production of tyrothricin in a synthetic medium supplemented with organic nitrogen compounds^{96,97,98,99,100}.

Several investigators have attempted to establish the biosynthetic pathways for the production of tyrothricin^{101,102,103,104}.

Akers, Lee and Lipmann have isolated two enzymes from Bacillus brevis that are responsible for the synthesis of the initial portion of the gramicidins²²³.

5.2 Isolation

Hotchkiss and Dubos have utilized solvent extraction to separate gramicidin and tyrocidine¹⁰⁵. Several patents have been issued on procedures for isolating gramicidin from fermentation broth^{106,107,108}.

5.3 Derivatives

Shepel and coworkers have reported the properties of analogs of gramicidin A with shorter peptide chains¹⁰⁹.

It has been found that the treatment of gramicidin with formaldehyde results in the formation of a compound with the same antibiotic activity but with much less hemolytic activity^{110,111,112,113,114}.

Various esters of gramicidin have also been prepared^{115,116,117}. Although these compounds have reduced hemolytic activity, they also possess less antibiotic activity.

6. Stability

6.1 Stability in Solution

Nitti and Nislo have shown that gramicidin is stable to autoclaving in aqueous solution¹¹⁸. Certain aqueous solutions were patented because they produced stable gramicidin solutions¹¹⁹.

Ishii and Witkop have found that treatment of gramicidin A with 1.5N hydrochloric acid in absolute methanol for one hour at room temperature selectively cleaved one peptide bond¹²⁰.

6.2 Effect of Light

The ultraviolet inactivation spectrum for gramicidin has been published by Setlow and Doyle¹²¹. Sugimoto and coworkers have presented infrared, ultraviolet, visible and ESR spectra for gramicidin solutions irradiated with various amounts of ultraviolet light¹²².

6.3 Stability of Formulations

Buckwalter has indicated that gramicidin solutions in propylene glycol and carbowaxes are stable to autoclaving¹²³. He also showed that the antibiotic can be solubilized in water with the aid of non-ionic wetting agents.

7. Analytical Methods

7.1 Identity Tests

Brustier and coworkers utilized the Adamkiewicz-Hopkins-Cole reaction as an identity test for gramicidin¹²⁴. The test detects the indole ring structure of the tryptophane residue.

Fischbach and Levine described the use of Ehrlich's reagent as an identity test for gramicidin¹²⁵. They also utilized a modified biuret reaction¹²⁵.

Ramachandran reported on the reaction of hydrazine with gramicidin to yield formic hydrazide¹²⁶. This product was detected by a color reaction.

7.2 Microbiological Assays

Microbiological assays are the primary assay method for antibiotics. They provide sensitive but non-selective methods. A variety of microbiological methods have been described for the assay of gramicidin. The official method in the United States is the turbidimetric method described in the Code of Federal Regulations^{127,128}.

7.21 Dilution Methods

Dilution assays are generally utilized as early microbiological methods before well defined standards are available. They have the advantage that one can compare the activity of one preparation to another without having a standard.

Reedy and Wolfson have described a tube dilution assay for gramicidin¹²⁹.

An agar dilution assay using Micrococcus lysodeikticus has been reported¹³⁰.

7.22 Dye Reduction Methods

The addition of a dye that is reduced by the growing microorganisms gives a microbiological assay a sharper end point.

Prévot reported on Janus green reduction methods with a number of different organisms^{131,132}.

DeFelip and coworkers use sodium resazurin as a dye to give a rapid assay in three to six hours¹³³.

7.23 Turbidimetric Assays

Cerioti has reported a turbidimetric assay using Micrococcus lysodeikticus¹³⁴.

Berridge and Barrett have reported an assay with Streptococcus agalactiae which can be read after $\frac{1}{2}$ hour incubation¹³⁵.

Kaiser and Camboni reported a turbidimetric assay utilizing Staphylococcus aureus¹³⁶.

Kramer and Kirschbaum have reported an assay with Streptococcus faecalis¹³⁷.

Leclercq has described a nephelometric assay for gramicidin^{138,139}.

Pain and coworkers have reported a turbidimetric assay utilizing lactic acid bacteria¹⁴⁰.

Kreuzig has described a turbidimetric assay utilizing Streptococcus faecalis¹⁴¹.

7.24 Agar Diffusion Assays

Since gramicidin is not very soluble in aqueous solution, relatively few agar diffusion assays have been reported.

Cerioti has reported an agar diffusion assay for gramicidin utilizing Micrococcus lysodeikticus¹³⁴.

Miller, Matt and Ciminera reported an agar diffusion assay for gramicidin¹⁴². Raitio and Bonn used this method to assay pharmaceutical preparations¹⁴³. The Swiss Pharmacopeia utilizes an agar diffusion assay with Sarcina lutea¹⁴⁴.

Viola and Canestrini reported on an agar well technique with Sarcina lutea¹⁴⁵.

Kreuzig has studied the agar diffusion assay for tyrocidine in detail using gel chromatography as an analytical technique¹⁴⁶.

7.25 Potentiation of Microbiological Assays

Nisonger reported that the addition of hexadecylpyridinium chloride to gramicidin potentiates the activity found by microbiological assay¹⁴⁷.

Forni found that traces of cobalt chloride enhances the activity of gramicidin toward Escherichia coli and Staphylococcus aureus¹⁴⁸.

Gillissen indicated that while cationic surfactants like cetylpyridinium chloride have a synergistic effect, Tween 80 inhibits the activity of gramicidin¹⁴⁹. This effect was confirmed by Barr and Tice¹⁵⁰.

Casilli and Ragni assayed gramicidin in the presence of cetrimide without interference using a cetrimide resistant strain of Staphylococcus aureus¹⁵¹.

7.3 Chemical Assays

7.31 Colorimetric Assays

Rittenberg and coworkers used a colorimetric assay for tryptophane to determine tyrothricin in fermentation broth¹⁵². Kreuzig described a semi-automated colorimetric assay for gramicidin utilizing the reaction of tryptophane with perchloric acid-butanol and ferric chloride^{141,153}.

Patel and Naravane have published an assay utilizing p-dimethylaminobenzaldehyde and nitrite¹⁵⁴. Ivashkiv has used this reaction to assay gramicidin and tyrocidine in fermentation broth¹⁵⁵. The antibiotics are separated by a simple solvent partition.

7.32 Spectrophotometric Methods

Oberzill has described a spectrophotometric assay for gramicidin¹⁵⁶. Thombs and coworkers have used the

absorbance of the peptide bond at 210 nm to measure gramicidin¹⁵⁷.

7.33 Miscellaneous Methods

White and Secor have measured gramicidin by measuring the Kjeldahl nitrogen¹⁵⁸.

7.4 Electrochemical Methods

Kramarczyk and Berg have described an indirect polarographic assay for gramicidin¹⁵⁹.

7.5 Hemolytic Methods

As has been mentioned previously, gramicidin has limited utility as an antibiotic because of its hemolytic activity (Section 1.0). Various workers have utilized the hemolytic property of the antibiotic as an assay tool. The hemolytic activity of gramicidin is probably due to its ability to form ion conducting channels in the membranes of red blood cells. The loss of isotonicity causes the cells to rupture.

Heilman and Herrell reported the first use of the hemolytic assay to the assay of gramicidin¹⁶⁰. Mann and co-workers compared the hemolytic activity of gramicidin and tyrocidine¹⁶¹. They reported that the addition of serum inhibits the hemolytic activity of gramicidin.

Dimick reported that the hemolytic assay can be used to measure the concentration of antibiotic in fermentation broth¹⁶². Other authors have reported on modified hemolytic methods^{163,164,165}.

7.6 Enzymatic and Other Biochemical Methods

Many antibiotics have been found to have inhibitory activity on enzyme systems. This inhibition can be utilized as the basis of an assay system.

Creaser reported that Staphylococcus aureus forms an inducible β -galactosidase¹⁶⁶. The production of this enzyme is inhibited by the addition of gramicidin. Strictly speaking, gramicidin does not inhibit the enzyme directly but this method could be used as the basis of a sensitive assay method.

Gramicidin was found to uncouple the phosphorylation of ADP from the enzymatic reduction of ferricytochrome C^{167,168,169,170,171,172,173,174,175}.

Hinkson reported that gramicidin inhibited the photoreduction of NAD by photosynthetic bacteria¹⁷⁶.

7.7 Chromatographic Methods

7.71 Counter Current Distribution

Gramicidin was first shown to be heterogeneous by counter current distribution²³. A water-methanol-chloroform-benzene (7:23:15:15) system was used to show the presence of two components. Craig later showed that gramicidin contained three crystalline components¹⁷⁷. Stamm discussed the application of counter current distribution to the separation of gramicidins¹⁷⁸. Ramachandran showed that there were at least four components in gramicidin and gave the fourth component the name gramicidin D³³.

Goss and Witkop separated each of gramicidins A,B and C into a pair of congeners and identified the major congener as valine-gramicidin and the minor component as isoleucine-gramicidin³⁹.

Okamoto and coworkers showed that gramicidin could be separated into three fractions (A, B and C) using droplet counter current distribution¹⁷⁹.

7.72 Paper Chromatography

Snell, Ijichi and Lewis published a series of paper chromatographic systems capable of separating various antibiotics including gramicidin¹⁸⁰. Detection was by bioautography. Forni and Cavalli used the following systems on Whatman No. 1 paper to distinguish between bacterial peptide antibiotics¹⁸¹.

t-Butanol-Acetic Acid-Water (74:3:25)

n-Butanol-Acetic Acid-Water (79:6:15)

Acetone-Water (70:30) + Ammonia

t-Butanol-Water (80:20) + Ammonia

Cunha and Baptista found that a butanol-acetic acid-water (50:25:25) system using Schleicher and Schull 2043a paper buffered to pH 3.0 gave the best separation of peptide antibiotics¹⁸². The same authors utilized salting out chromatography to separate gramicidin and tyrothricin¹⁸³.

Singh utilized anionic dyes to detect gramicidin on paper chromatograms¹⁸⁴. Paris and Theallet described three paper chromatographic systems for gramicidin¹⁸⁵. Ritschel and Lercher described two solvent systems for antibiotics¹⁸⁶. The solvent systems were butanol-pyridine-acetic acid-water (15:10:3:12) and water saturated butanol-water saturated ethyl ether-acetic acid (5:1:1) on Schleicher and Schull 2043b paper. The antibiotics were visualized by ninhydrin.

DeFranca and coworkers described a system of 25 ml 9:1 acetone-water, 5 ml chloroform, 2 ml ethylene glycol and 1 ml of pyridine¹⁸⁷. They claim the method is as accurate as the microbiological assay.

7.73 Thin Layer Chromatography

Nussbaumer utilized a solvent system of butanol-acetic acid-water (10:1:3) on acid Silica gel G¹⁸⁸. Pitton described the following five thin layer systems for several antibiotics including gramicidin¹⁸⁹.

Water-Methanol-Butanol-Butyl Acetate-Acetic Acid
(12:2.5:7.5:40:20)

Water-Butanol-Pyridine-Acetic Acid (14:30:20:6)

Water-Butanol-Pyridine-Acetic Acid (16:40:8:16)

Water-Butanol-Acetic Acid (39:55:6)

Aqueous phase of Methanol-Ammonia-Chloroform
(10:10:20)

Guven and Ozsari reported some thin layer systems to use as identity tests for antibiotics including gramicidin¹⁹⁰. McGilveray and Stickland described two thin layer chromatographic systems to use to identify several antibiotics including gramicidin¹⁹¹.

Nekola published a thin layer chromatographic assay for gramicidin in fermentation broth¹⁹². The

broth was adjusted to pH 4.5 with HCl and the antibiotics precipitated. The residue was dissolved in methanol and purified on an alumina column. The eluate was chromatographed on silica gel using water-methanol-butanol-butyl acetate-acetic acid (24:5:15:80). The absorbance of the gramicidin spot was then determined.

Kreuzig reported a high performance TLC method for gramicidin in fermentation broth^{193,194}. A Kieselgel 60 plate is developed with acetic acid-butyl acetate-butanol-methanol-water (40:80:15:5:12). The gramicidin spot was visualized by spraying with ethanolic 4-dimethylaminobenzaldehyde-HCl and quantitated by scanning at 570 nm.

7.74 Electrophoresis

Cunha and Baptista reported an electrophoresis method to determine gramicidin in pharmaceutical preparations¹⁹⁵. Paris and Theallet have described two paper electrophoresis systems for gramicidin¹⁸⁵. Cunha and Gomes have published paper electrophoresis methods for various peptide antibiotics¹⁹⁶. Lightbown and DeRossi utilized agar electrophoresis to identify various antibiotics including gramicidin¹⁹⁷.

7.75 Column Chromatography

Moses has patented a process for the separation of gramicidin from tyrothricin¹⁹⁸. The 80% aqueous methanol solution is passed through a cation ion exchange resin in the hydrogen cycle followed by an anion resin in the hydroxyl cycle.

Bartley and coworkers separated gramicidin from tyrocidine using Sephadex LH20¹⁹⁹.

7.76 High Performance Liquid Chromatography

Axelsen and Vogelsang have reported the separation of gramicidins A, B and C by HPLC using Zorbax ODS (5 μ m) eluted at 60°C with methanol-5 mM ammonium sulfate (37:13)²⁰⁰.

8. Reviews

A large number of general reviews have been written on gramicidin^{201,202,203,204,205,206,207,208,209,210,211,212,213,214,215,216,217,218,219,220,221,222}.

These reviews cover the chemical properties, biological properties and medical use of gramicidin.

A review on the analytical methods for gramicidin and other antibiotics has been prepared by Brewer and Platt²²⁴.

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GRISEOFULVIN

Edward R. Townley

1. Description
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 - 7.094 Gas Chromatography
 - 7.095 High Performance Liquid Chromatography
 - 7.10 Biological Methods of Analysis
8. Identification and Determination in Body Fluids
9. Analysis of Dosage Forms
10. Acknowledgments

1. Description

1.1 Name, Formula, Molecular Weight

Chemical Names

(2S-trans)-7-Chloro-2',4,6-trimethoxy-6'-methylspiro [benzofuran-2(3H), 1'-(2) cyclohexene]3,4'-dione

7-chloro-4,6-dimethoxycoumaran-3-one-2-spiro-1'-(2'-methoxy-6'-methylcyclohex-2'-en-4'-one)

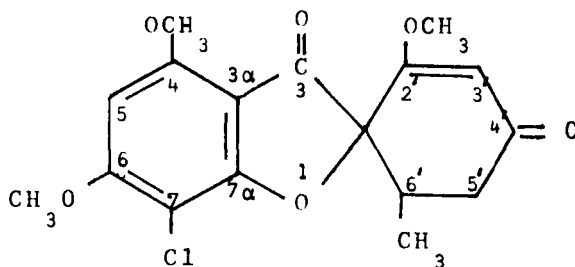
Generic Names

Griseofulvin

Trade Names

Fulcin; Fulvicin U/F, Fulvicin P/G, Grifulvin; Grisactin; Grisovin, Gris-PEG, Grysio, Lamoryl, Likuden, Neo-Fulcin, Poncyl; Spirofulvin; Sporostatin.

Formula and Molecular Weight



$C_{17}H_{17}ClO_6$

Molecular Weight 352.77

1.2 Appearance, Color, Odor

Griseofulvin is a white, odorless, crystalline powder.

1.3 Compendial References, Other

Griseofulvin is listed in the following compendia: The United States Pharmacopia (1) The British Pharmacopia (2) The European Pharmacopia (3) and the Merck Index (4). A previously published review (5) is a good source for physical and chemical data, production, use, occurrence and biological information.

2. Physical Properties

Physical measurements by Schering Corporation are provided for Batch Number UGFP-1961. This batch is chromatographically pure with the exception of 1.1% dechlorogriseofulvin.

2.01 Circular Dichroism Spectra

The circular dichroism spectra (Figure 1) was obtained on a 0.0155 mg/ml solution in methanol with a Cary Model 61 Circular Dichroism Spectrophotometer. The following molar ellipticity values were obtained:

Table I

<u>Wavelength</u> <u>nm</u>	<u>Molar</u> <u>Ellipticity</u> <u>[θ]</u>
345 shoulder	+ 7,520
326 shoulder	+ 19,100
314 shoulder	+ 23,200
294 peak	+ 43,700
236 peak	+110,000
218 peak	- 97,500

2.02 Nuclear Magnetic Resonance Spectra

The proton NMR spectrum of griseofulvin (Figure 2) was obtained in DMSO- d_6 solution (conc. w/v = 10 mg/0.40 ml) containing TMS as internal reference utilizing the Varian Associates CFT-20 Spectrometer operating at a frequency of 79.5 MHz. The chemical shifts (δ , ppm) are with reference to TMS. The experimental conditions are:

Sweep width	=	800 Hz
Pulse width	=	6.0 μ sec (25°tip)
Acquisition time	=	5.1 sec
Data table	=	8 K

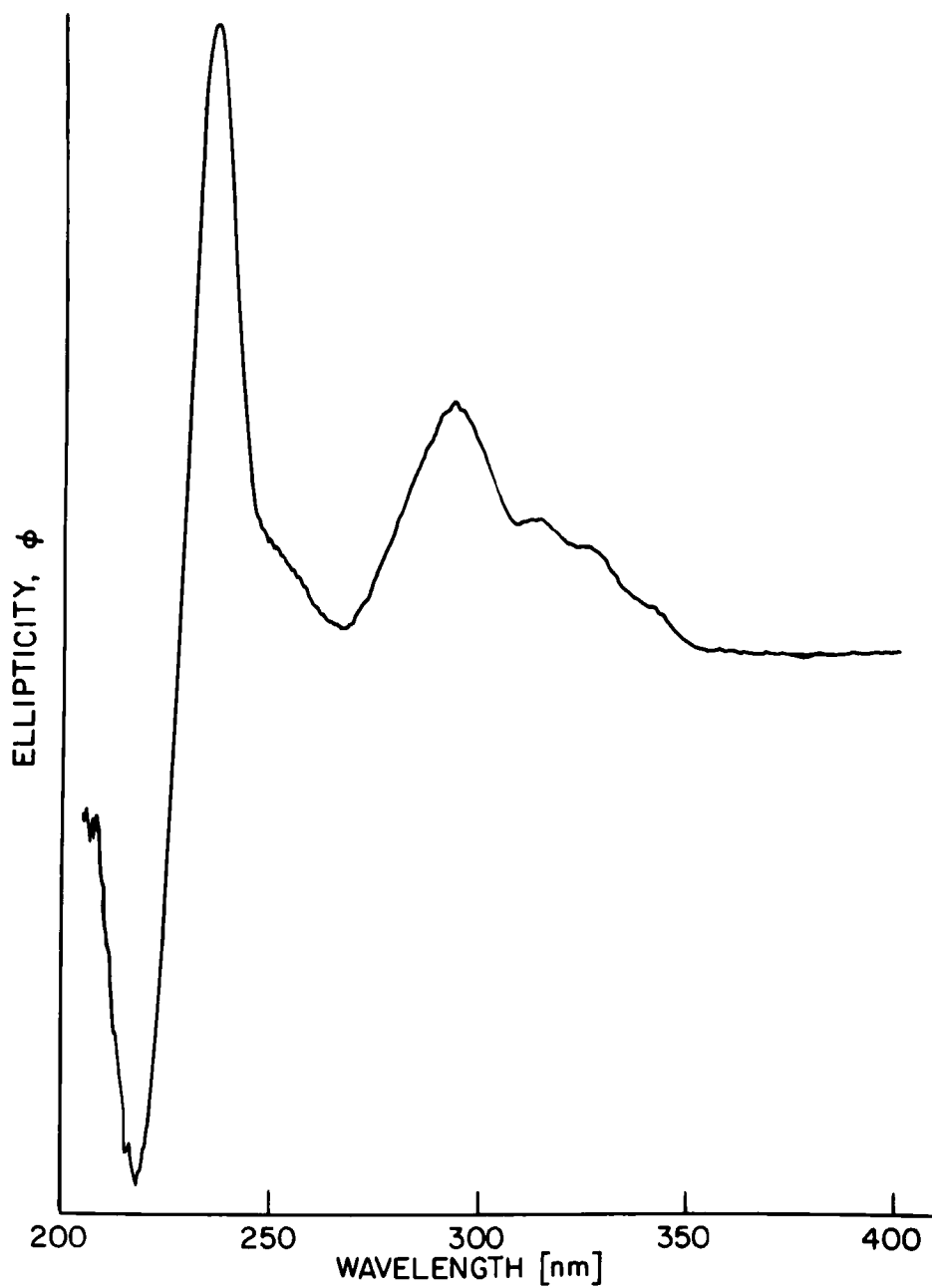


FIGURE 1: Circular Dichroism Spectra of Griseofulvin

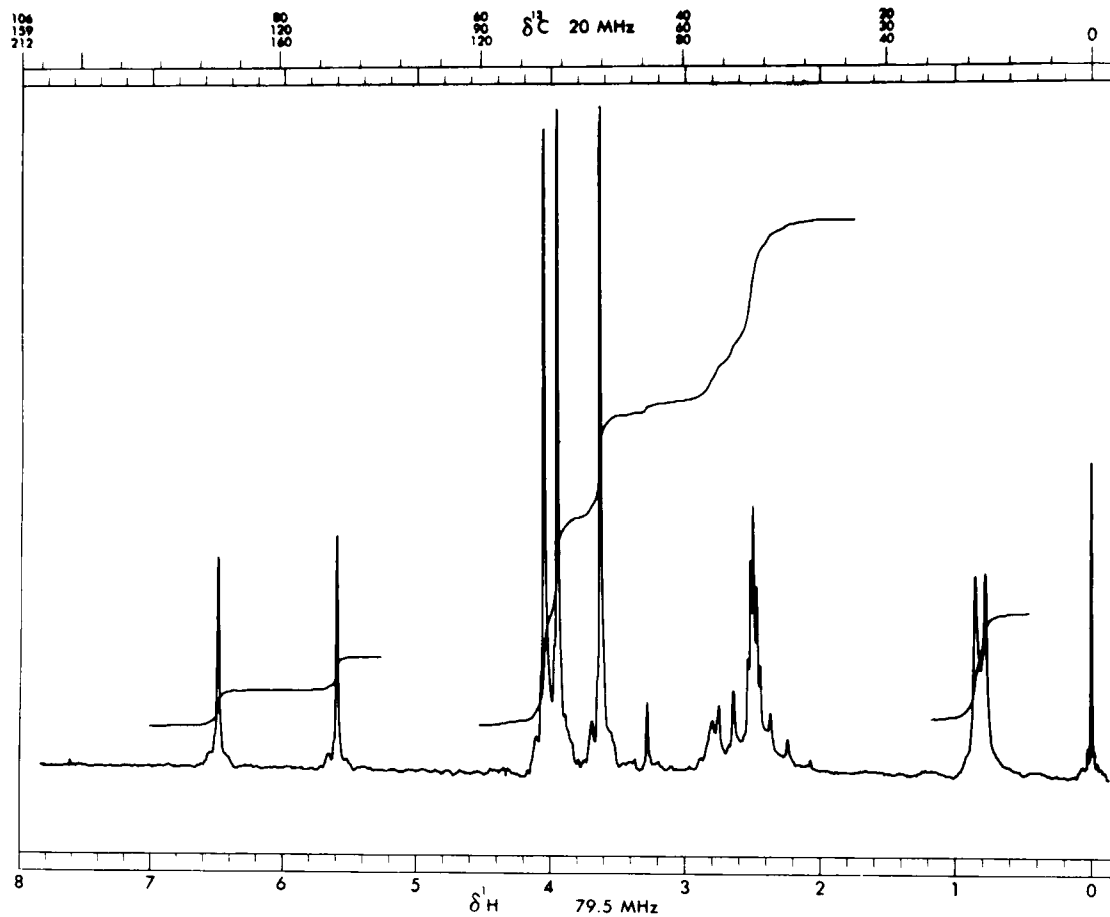


FIGURE 2: Proton Nuclear Magnetic Resonance Spectra of Griseofulvin in DMSO d₆

Table II

<u>Proton</u>	<u>Chemical Shifts (δ, ppm)</u>		
6'-CH ₃	0.82	d(J=6.5 Hz)	(3H)
5'-CH ₂ } 6'-CH ² }	2.3-2.9	m	(3H)
2'-OCH ₃	3.63	s	(3H)
4-OCH ₃ } 6-OCH ₃ }	3.96 4.04	s s	(3H) (3H)
3'-H	5.59	s	(1H)
5-H	6.50	s	(1H)

d= doublet, s= singlet, m= multiplet.

The carbon-13 NMR spectrum of griseofulvin (Figure 3) was obtained at ambient temperature in DMSO-d₆ containing TMS as internal reference utilizing Varian Associates XL-100-15 spectrometer equipped with Fourier accessories. The system was locked to the deuterium resonance frequency of the solvent, and operated at a frequency of 25.2 MHz for carbon-13. The chemical shifts are reported (c, ppm.) from the internal standard TMS.

Sweep width = 5500 Hz
Pulse width = 15 μ sec (66° tip)
Acquisition time = 1.6 sec
Acquisition delay = 0.20 sec

Table III

<u>Carbon</u>	<u>Chemical Shift (δ_c)</u>	<u>Carbon</u>	<u>Chemical Shift (δ_c)</u>
1'	90.11	6 OCH ₃	56.52
2'	170.22	3	191.12
3'	104.60	3a	104.04
4'	195.45	4	157.59
5'	39.49	5	91.27
6'	35.52	6	164.44
6' CH ₃	13.77	7	95.24
4' OCH ₃	57.54	7a	168.57
2' OCH ₃	56.97		

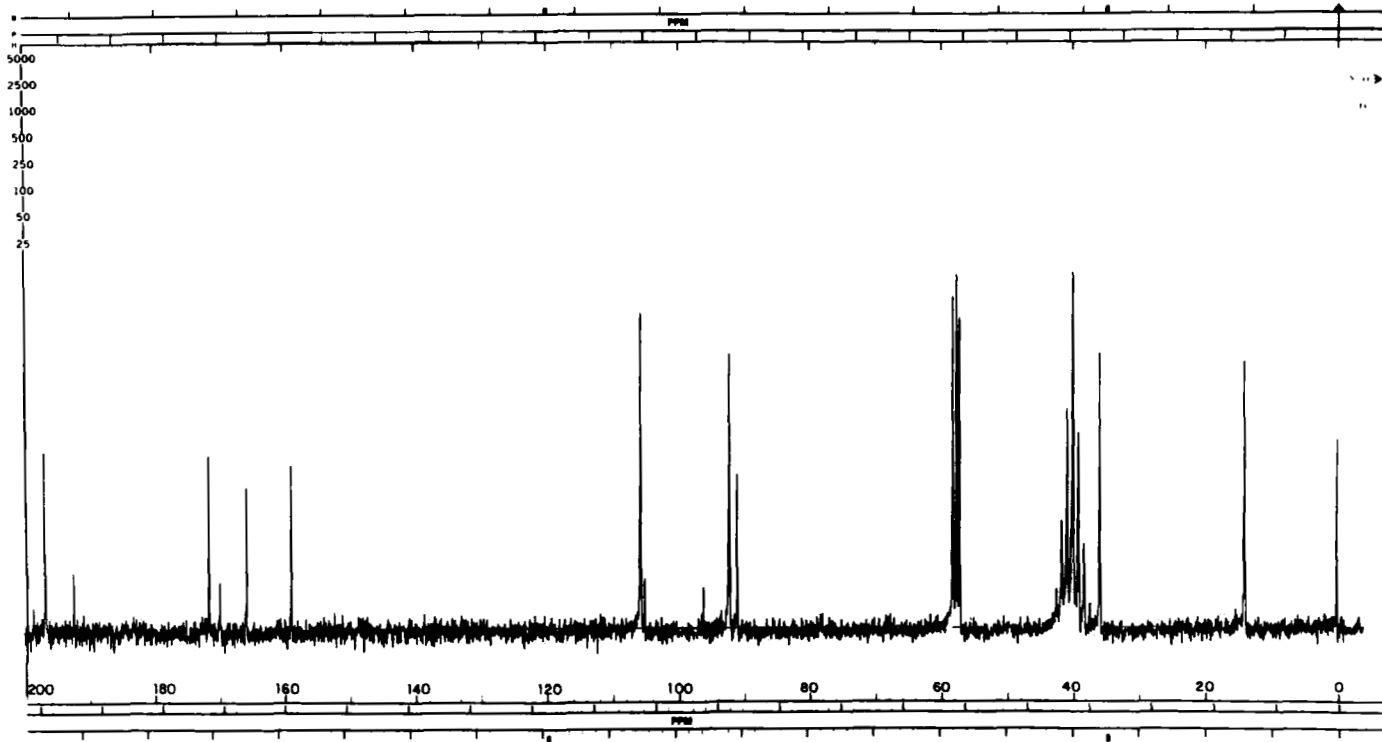


FIGURE 3: Carbon 13, Nuclear Magnetic Resonance Spectra of Griseofulvin in $\text{DMSO}-d_6$

The spectrum is in substantial agreement with the data reported by Wenkert et. al, (6). However, on the basis the proton coupled carbon 13 NMR spectra, the assignments for 6 and 7 α are reversed [Brambilla (16)] from those previously reported. The new assignments are based on long range H-C-O-C couplings.

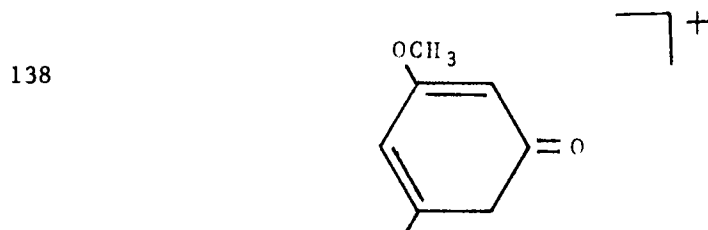
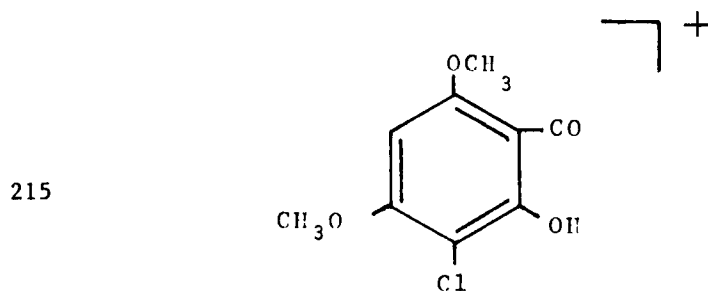
2.03 Mass Spectrum

The medium resolution, electron-impact mass spectrum of griseofulvin (Figure 4) was run on a Varian-Mat CH-5 Mass Spectrometer. Instrumental conditions were; Electron Energy 70eV; Source Temperature 250°C; Sample Probe Temperature 140°C.

The fragmentation ions, given below, are consistent with the griseofulvin structure.

Table IV

Mass (amu)	Ions	Losses
352	M ⁺	
337	(M-15) ⁺	CH ₃
321	(M-31) ⁺	CH ₃ O
310	(M-42) ⁺	CH ₂ CO
284	(M-68) ⁺	MeC=CHCO



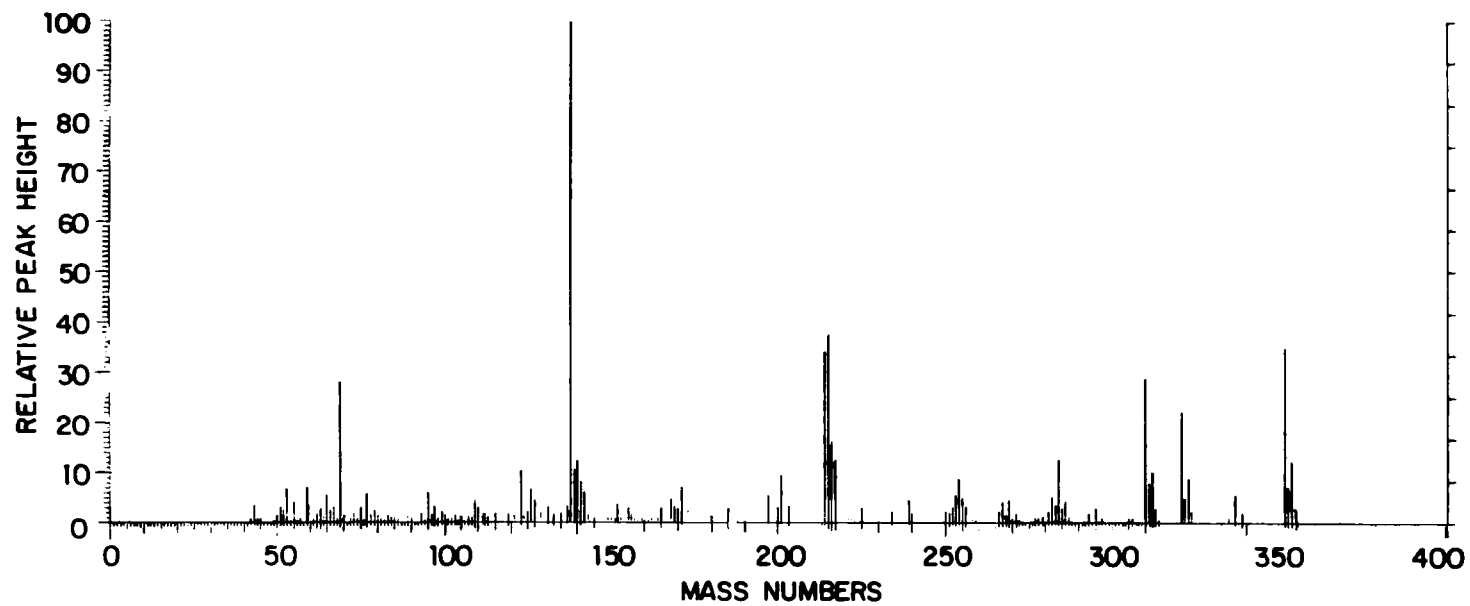
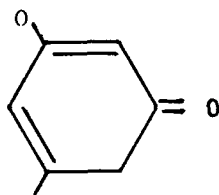


FIGURE 4: Mass Spectrum of Griseofulvin

123



2.04 Ultraviolet Spectrum

The ultraviolet spectrum of griseofulvin in anhydrous methanol solution at 25°C gave the following absorptivity values

Wavelength maximum is at 324 nm; $a = 15.5$

Wavelength maximum is at 291 nm; $a = 68.3$

Wavelength maximum is at 235 nm; $a = 64.0$

The ultraviolet spectrum is shown in Figure 5.

2.05 Infrared Spectrum

The infrared spectrum of griseofulvin, obtained as a mineral oil mull, was run on a Perkin-Elmer Model 180 grating IR spectrophotometer. Important absorption assignments are given in Table V. The spectrum is given in Figure 6.

Table V

<u>Wavenumber (cm⁻¹)</u>	<u>Assignment</u>
1703 (s)	C=O stretch; benzofuranone ring carbonyl
1658 (s)	C=O stretch; cyclohexenone carbonyl
1615, 1597, 1580 (s)	C=C stretch, aromatic and cyclic unsaturation
1501 (m)	C=C stretch, aromatic
1220, 1210 (s)	C-O stretch, aryl methoxyl

Intensity

s - strong

m - medium

2.06 X-ray Diffraction

The X-ray diffraction pattern of griseofulvin was obtained on a Phillips ADP-3500 X-ray Diffractometer using Cu K α radiation (1.5405Å) and Ni filter. The data is given in Table VI.

2.06 X-Ray Powder Diffraction
Pattern of Griseofulvin

Table VI

<u>2θ</u>	<u>d(A)^o *</u>	<u>I/I' **</u>
4.009	22.038	18
4.117	21.460	14
10.679	8.284	46
13.123	6.746	48
13.844	6.397	8
14.497	6.110	59
16.422	5.398	100
17.669	5.020	7
19.194	4.624	30
19.625	4.523	16
20.184	4.399	24
21.562	4.121	34
21.986	4.043	23
22.462	3.958	45
23.765	3.744	72
24.067	3.698	10
24.295	3.663	13
25.780	3.456	26
26.567	3.355	87
28.418	3.141	57
29.835	2.995	29
31.104	2.875	20
31.185	2.868	19
31.385	2.850	24
32.624	2.745	14
32.674	2.741	14
34.852	2.574	12
34.914	2.570	11
35.896	2.502	18
36.142	2.485	14
36.202	2.481	15
36.290	2.475	14
37.077	2.425	14
37.270	2.413	13
38.500	2.338	18
38.569	2.334	18
38.659	2.329	19

*d (interplanar distance) = $n / 2 \sin \theta$

**I/I' = relative intensity (based on the highest intensity of 100)

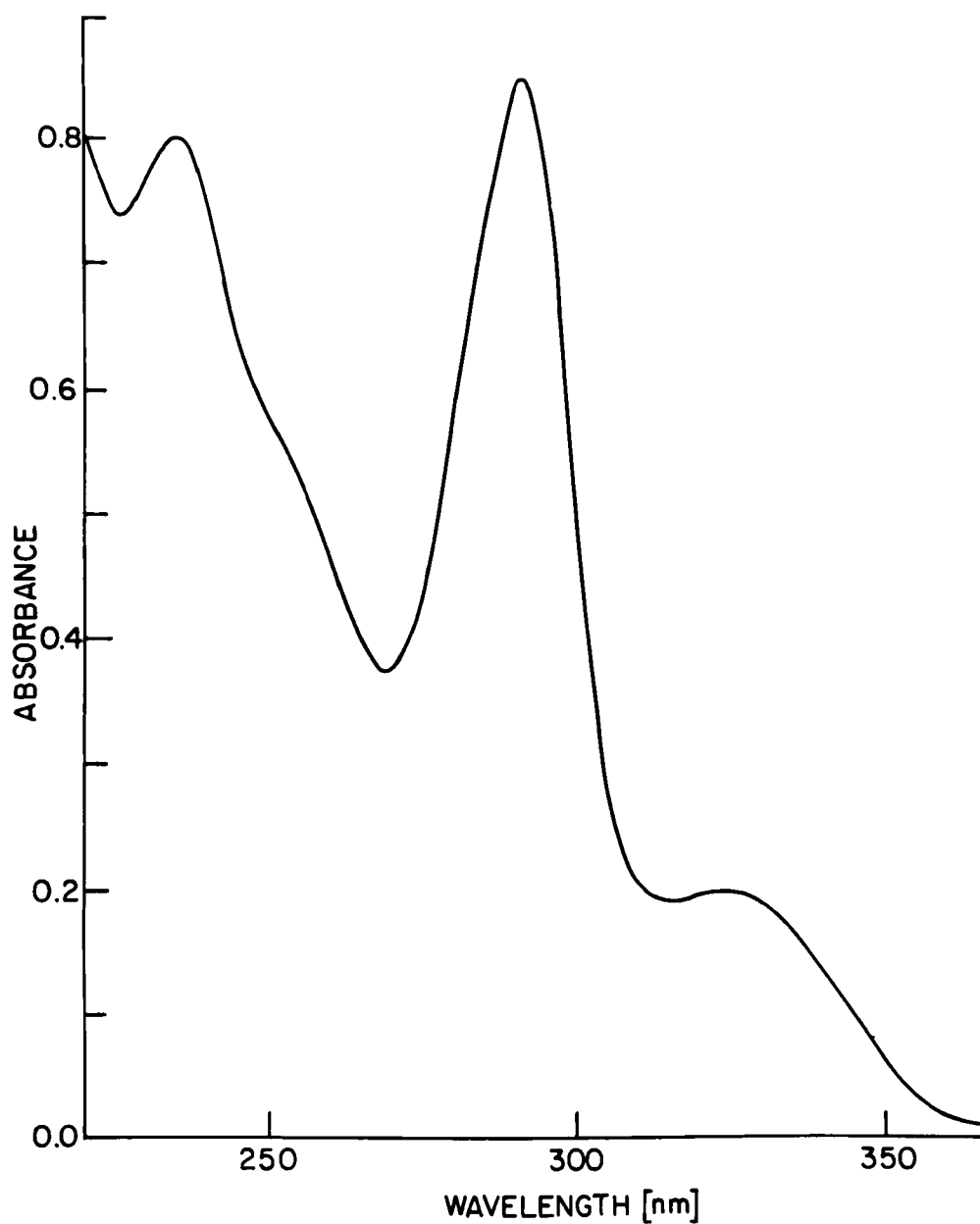


FIGURE 5: Ultraviolet Spectrum of Griseofulvin Obtained in Anhydrous Methanol Solvent

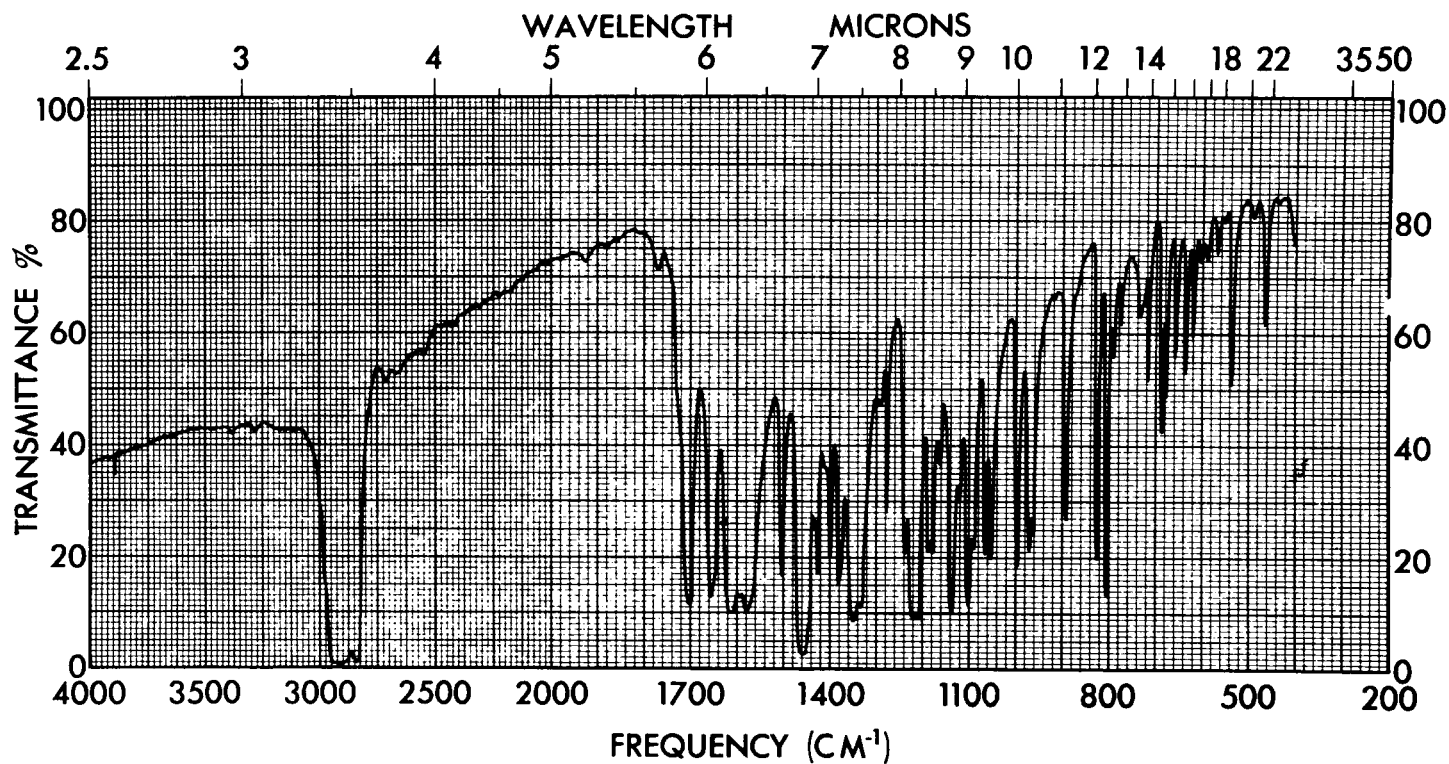


FIGURE 6: Infrared Spectrum of Griseofulvin Obtained as a Mineral Oil Mull.

2.07 Fluorescence and Luminescence

Griseofulvin exhibits both fluorescence and luminescence. A report by Neely et al., (7) gives corrected fluorescence excitation (max. 295 nm) and emission (max. 420 nm) spectra, values for quantum efficiency of fluorescence (0.108) calculated fluorescence lifetime (0.663 nsec) and phosphorescence decay time (0.11 sec.). The fluorescence excitation and emission spectra are given in Figure 7.

2.08 Photolysis

There is no change in the thin layer chromatogram (single spot) or in the fluorescence or ultraviolet spectra after irradiation in methanol with a xenon lamp for 20 hours. It is therefore concluded that there is no significant photodegradation of griseofulvin under reasonable conditions of light exposure (7).

2.09 Optical Rotation

Griseofulvin exhibits the following optical rotation when dissolved in these solvents.

Table VII

Saturated chloroform	$[\alpha]_D^{17} = +370$	4
Acetone	$[\alpha]_D^{21} = +337$	5
Dimethylformamide	$[\alpha]_D^{26} = +358$	17
Dioxane	$[\alpha]_D^{26} = +302$	17

2.10 Melting Range

The Differential Thermal Gravimetry curve (Figure 9) demonstrates that the griseofulvin melting point takes place with decomposition. The melting range of griseofulvin from several sources is given in Table VIII.

Table VIII

Melting Range °C	<u>Reference</u>
218 to 224	3
220	4
218	17
217-224	51

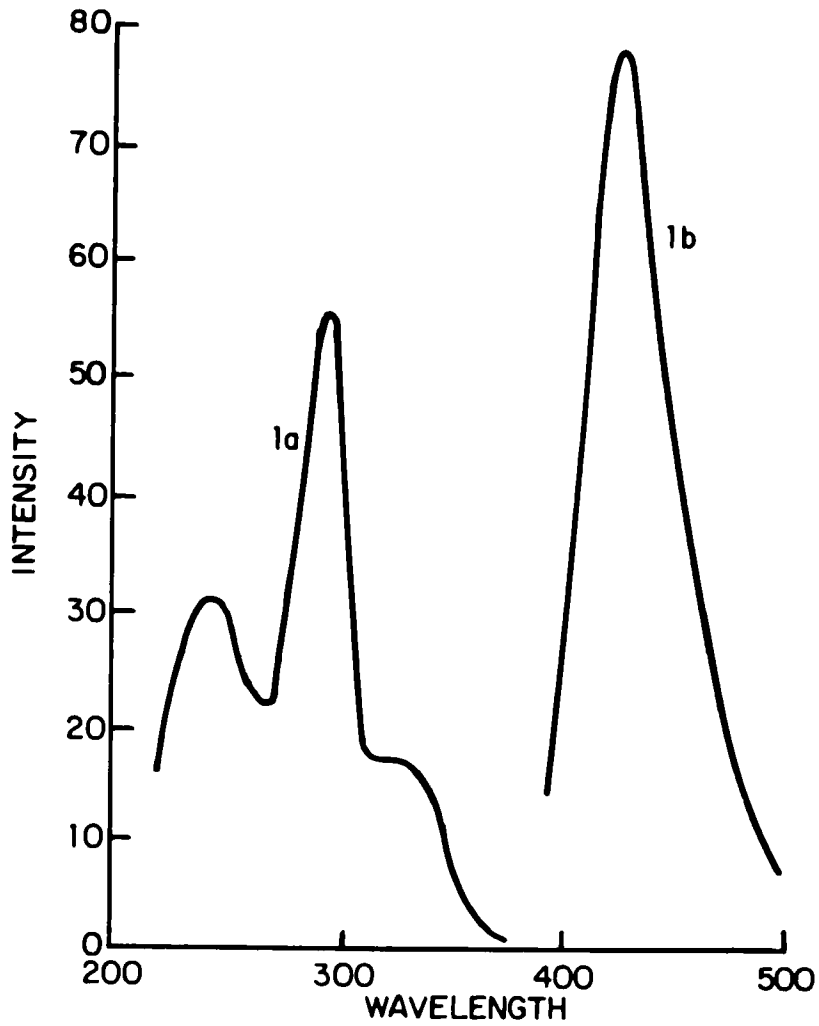


FIGURE 7: Corrected Fluorescence and Emission Spectra of Griseofulvin: 1a, Excitation Spectrum with Emission at 420 nm; 1b, Emission Spectrum with Excitation at 295 nm.

2.11 Differential Scanning Calorimetry

Figure 8 shows the DSC thermogram of griseofulvin obtained with a DuPont Model 900 Thermal Analyzer. A single sharp melting endotherm occurs for this substance with onset temperature at 216°C.

2.12 Thermogravimetry

Figure 9 shows the TG thermogram of griseofulvin obtained with a DuPont Model 950 Thermogravimetric Analyzer. The thermogram shows no weight loss from ambient to about 200°C followed by weight loss due to sublimation.

2.13 Electrophoretic Properties

Zeta potentials of dispersed griseofulvin have been studied both alone, and in the presence of surface-active agents, the latter at a controlled pH (8). A Mobility/Zeta Potential pH plot of = +25mV griseofulvin, shows a positive charge at pH 1.5 which rapidly decreases to zero at pH 2.4. There is then reversal of charge followed by an increase over the pH range 2.4 to 7.0. The zeta potential at the latter pH is -45 mV. The potential then stays constant over the pH range 7 to 10.

2.14 Solubility

The following data are given for the solubility of griseofulvin at 25°C; acetone 30 g/L, carbon tetrachloride 2 g/L, dichloroethane 80 g/L, dimethylacetamide, 40 g/L, dioxane 30 g/L, ethyl ether 0.7 g/L, heptane 0.3 g/L, methanol 0.4 g/L; mineral oil <0.1 g/L; propylene glycol 2 g/L; Span 80 0.2 g/L, Tween 80, 7 g/L water 0.2 g/L (17).

3. Production and Synthesis

Griseofulvin is biosynthetically manufactured by elaboration with *Penicillium griseofulvum* and related strains of *Penicillia*. The biosynthesis has been the subject of numerous chemical and biological studies, the latest of which is given by Harris, et. al. (9) Figure 10. Other proposed biosynthetic pathways are discussed.

Griseofulvin was first isolated in 1938 by Oxford (10) et. al., (1939); its total synthesis was accomplished in 1960 and following years in several laboratories (Brossi et al., 1960 (11) Grove, 1963; (12) Mutant strains of *P. patulum* are used for the commercial production of the antibiotic by fermentation (9).

4. Impurities

Some fermenter broth impurities have been listed by Holbrook, Bailey and Bailey (13) and repeated in a description

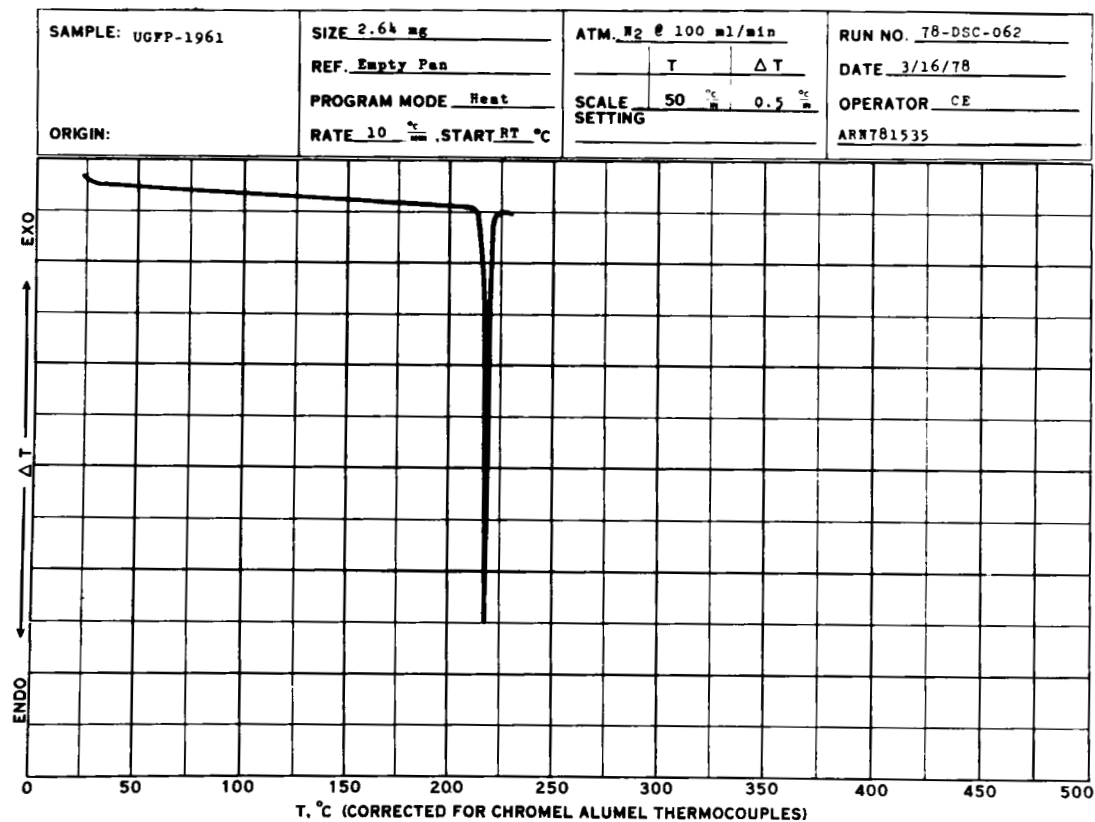


FIGURE 8: Differential Scanning Calorimetry Curve of Griseofulvin

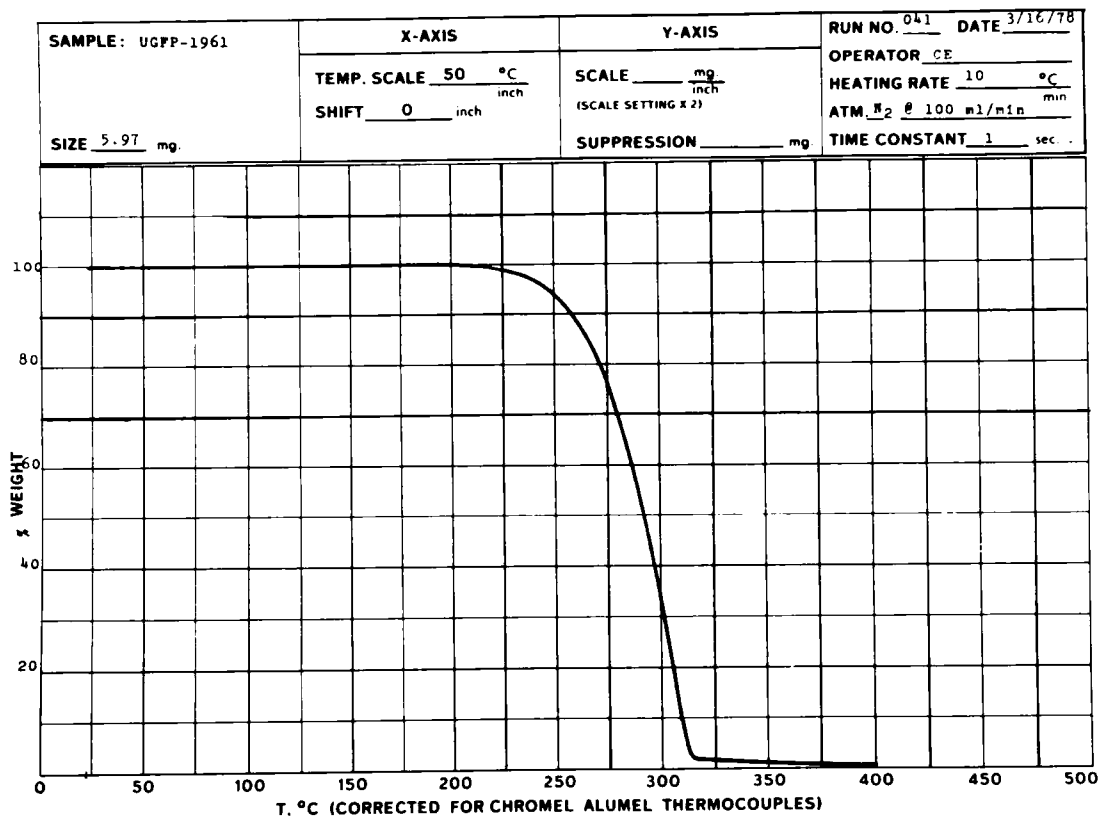


FIGURE 9: Thermogravimetry Curve of Griseofulvin

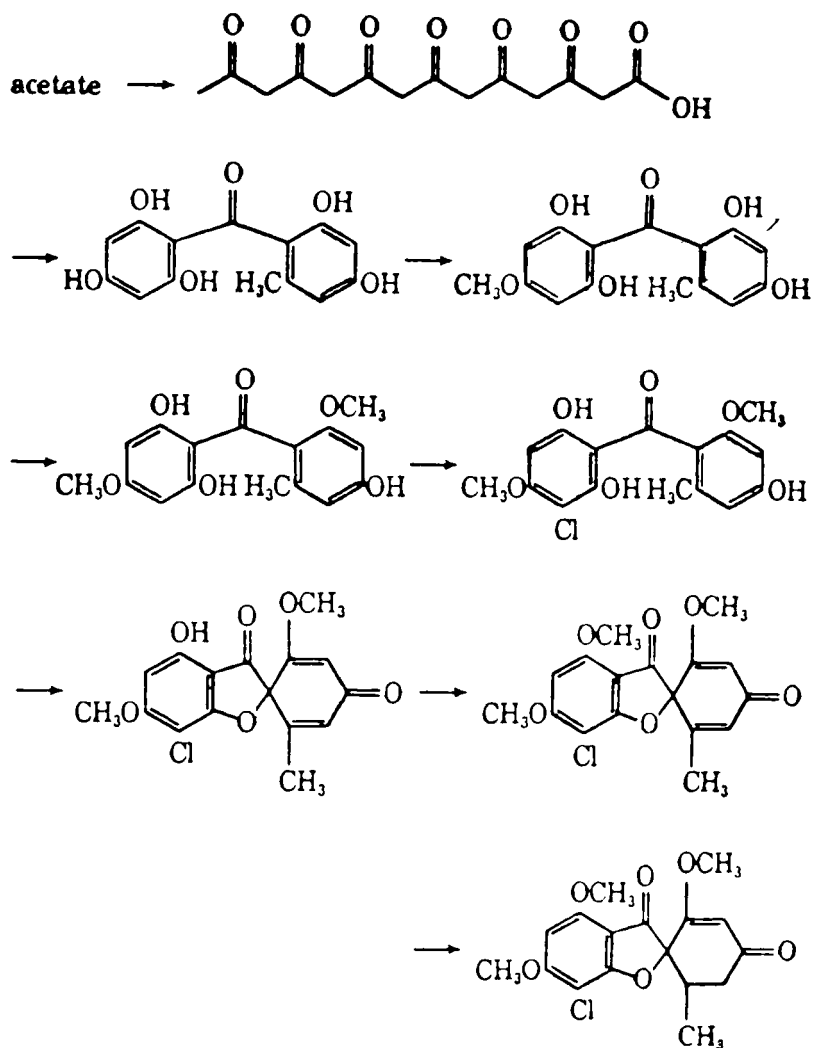


FIGURE 10: A Biosynthetic Route for Griseofulvin.

of efficient liquid chromatographic separation systems by Bailey and Brittain (14) and in a gas chromatographic separation system by Margosis (15). Structures are in Figure 11.

The common impurity found in commercial batches of griseofulvin is dechlorogriseofulvin (14,15) which appears to be in the range of 0.5 to 3.5%.

5. Stability

Griseofulvin is a stable drug substance. After 12 years storage at room temperature no decomposition was detected by differentiating LC methods (16). There is no photodegradation under reasonable conditions of light exposure (7). Griseofulvin is converted to griseofulvic acid under acidic conditions.

6. Drug Metabolic Products

The major human metabolite of griseofulvin is 6-demethylgriseofulvin and its glucuronide (17,18) which account for about 65% of the intravenous dose (19) and 35 to 65% of the oral dose (20,21). The 6-demethylgriseofulvin is also the major metabolite in dogs (22) and rabbits (23) while both 4-demethylgriseofulvin and 6-demethylgriseofulvin are major metabolites in rats (24) and mice (25). These metabolites can be determined by gas liquid chromatography via isopropoxyl derivatives (18) or trimethylsilyl ether derivatives (26,27). The 6-demethylgriseofulvin has been measured in urine by high performance liquid chromatography (28) and ultraviolet spectrophotometry (19). Only trace amounts of griseofulvin are found in the urine (28).

7. Methods of Analysis

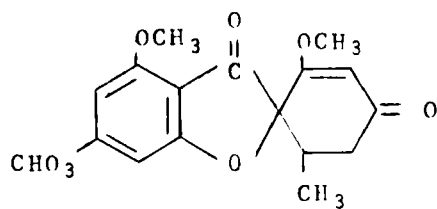
7.01 Identification

A wine red color is produced when about 5 mg of griseofulvin are dissolved in 1 ml of sulfuric acid with about 5 mg of powdered potassium dichromate (29).

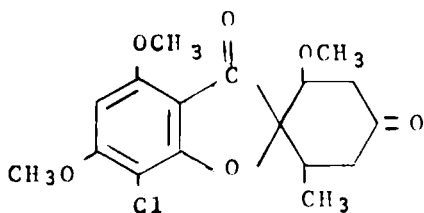
7.02 Elemental Analysis

Analysis of griseofulvin, was determined for carbon, hydrogen, and chlorine. The carbon, and hydrogen analysis was performed on a Perkin Elmer Model 240 instrument. Analysis for chlorine was performed by combustion of the sample and coulometric titration using an American Instrument Co. Chloride Titrator.

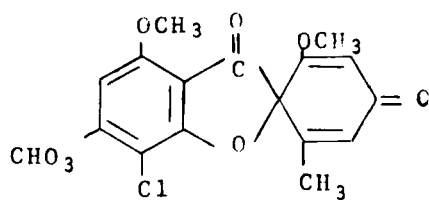
The results from the elemental analysis are listed in Table IX.



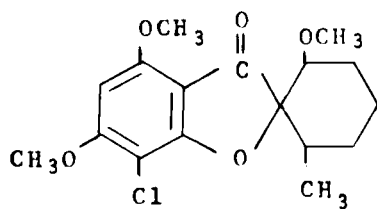
Dechlorgriseofulvin



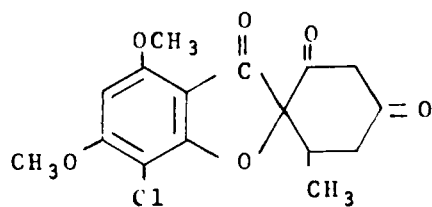
Dihydrogriseofulvin



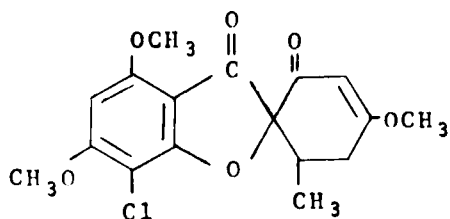
Dehydrogriseofulvin



Tetrahydrogriseofulvin



Griseofulvic Acid



Isogriseofulvin

FIGURE 11: Impurities Found in the Fermenter Broth

Table IX

Elemental Analysis of Griseofulvin: Batch UGFP-1961

<u>Element</u>	<u>% Theory</u>	<u>% Found</u>
C	57.88	57.97
H	4.86	4.84
Cl	10.05	9.92

7.03 Spectrophotometric Analysis

Quantitative ultraviolet analysis of griseofulvin may be performed by comparison to a Reference Standard. The ultraviolet absorbance is described in Section 2.04 and Figure 5.

7.04 Spectrofluorometric Analysis

Griseofulvin exhibits fluorescent properties which have been utilized for highly sensitive analyses in blood and serum (30-33) skin and sweat (34). Riegelman (32) has combined TLC separation with a fluorimetric densitometer readout to give a highly specific and sensitive griseofulvin determination in plasma. Other analyses are commonly performed in either 1% aqueous ethanol (30), activation maxima 295 and 335 nm, fluorescence maxima at 450 nm or anhydrous methanol (31) activation maxima unchanged at 295 and 335 nm, fluorescence maxima 420 nm. Values are uncorrected. Other applications to the analysis of bulk drugs, dosage forms or as a detection method for high performance liquid chromatography are feasible.

7.05 Colorimetric Analysis

A colorimetric assay of griseofulvin, based on the yellow-orange color ($\lambda_{\text{max}}=420$ nm) which develops when griseofulvin is heated with isonicotinic acid hydrazide in alkaline medium has been described by Unterman (35) and the mechanism investigated by Unterman and Duca (36).

7.06 Iodometric Analysis

Iodometric analysis has been applied to the determination of griseofulvin in stages of the manufacturing process (37). The mycelium is extracted with chloroform and the analysis carried out in alcoholic solution. The 0.01N iodine solution is standardized with griseofulvic acid.

7.07 Turbidimetric Analysis

A turbidimetric assay for potency evaluation of griseofulvin has been reported (38). The drug is dissolved in

ethylene glycol monomethyl ether (methyl cellosolve). Polymerization is induced with glycerol and guanosine-5'-triphosphate (GTP).

7.08 Polarographic Analysis

A study directed toward a comparison of the reduction potentials for griseofulvin homologs and analogs suggests that polarography is a method of griseofulvin identification (39). In ethanolic solution with 0.2 M KCl supporting electrolyte, griseofulvin shows two polarographic waves with half-wave potentials at about -1.58 V and -1.84 V. This system has been applied with good results to the determination of finished products including tablets. The accuracy, precision and selectivity of the method was compared with the iodometric method (40). Isogriseofulvin and griseofulvic acid do not interfere. (41,42)

7.09 Chromatographic Analyses

7.091 Partition Column Chromatography

Griseofulvin maybe separated from observed structurally similar impurities in the fermenter broth by means of partition column chromatography (13). A Celite column packing and solvent system consisting of methanol:water:hexane:chloroform (8:2:9:1) was used. Tetrahydrogriseofulvin, dihydrogriseofulvin, isogriseofulvin, dechlorogriseofulvin are separated from griseofulvin. The structures for these compounds have been given in Section 5.

7.092 Paper Chromatography

A paper chromatography system is given in Table X. (43)

Table X

<u>Solvent System</u>	<u>Paper</u>	<u>Detection</u>	<u>Reference</u>
Benzene:Cyclohexane; Methanol:Water (5:5:6:4) Glacial acetic acid, 0.5%, was added to the organic phase of the solvent after equilibration.	Whatman No. 1	UV	43

7.093 Thin Layer Chromatography

Thin layer chromatographic systems are given in Table XI. The detection method was U.V.

Table XI

<u>Solvent System</u>	<u>Adsorbent</u>	<u>Tr</u>	<u>Reference</u>
Methanol:n-butanol; 95% ethanol:conc. ammonium hydroxide (4:1:2:1, by vol.)	Silica Gel	0.64	44
Chloroform:isopropanol (3:1, by vol.)	Silica Gel	0.86	44
n-butanol:formic acid: water (77:10:13 by vol.)	Silica Gel	0.86	44
n-Butanol:95% ethanol conc. ammonium hydroxide:water (4:1:2:1 by vol.)	Silica Gel	0.64	44
Chloroform:acetone (93:7 by vol.)	Silica Gel	0.65	45
Chloroform:methanol (10:1 by vol.)	Silica Gel	0.82	46
Chloroform:acetic acid diethyl ether (17:1:3)	Silica Gel	-	47
Methanol:benzene (2:98 by vol.)	Silica Gel	0.50	48
Ethyl acetate	Silica Gel	0.50	17

7.094 Gas Chromatography

Successful griseofulvin analyses by gas chromatography are reported for simulated samples (49) fermentation extracts (45) and pharmaceutical bulk and dosage forms (50). Margosis (15) describes the application of gas chromatography to the purity determination of griseofulvin. Separation from the related compounds; dehydrogriseofulvin, iso-griseofulvin and dechlorogriseofulvin was demonstrated. In a subsequent collaborative study (50) the accuracy and precision of the method was established. Although griseofulvin is thermally stable, the high GLC temperatures require precautions similar to those taken for steroids when preparing columns, column supports and associated equipment (45).

The specific and sensitive GLC determination of griseofulvin in body fluids and tissues such as skin, sweat, urine and plasma with electron capture detection has been used by several investigators (34,51,52).

Gas chromatographic conditions are given in Table XII.

Table XII

<u>Column</u>	<u>Carrier Gas</u>	<u>Column Temp.</u>	<u>Internal Standard</u>	<u>Reference</u>
150 cm x 4 mm I.D.; U-shaped stainless steel tubing, 1.5% QF-1 on Anakrom ABS	N ₂	230 ^o	diphenyl-phthalate	45,49
3 ft x 4 mm I.D.; coiled glass tubing, 3% OV-101 on Gas Chrom Q	He ₂	245 ^o	tetraphenyl-cyclopentadienone	15
5 ft x 4 mm I.D.; glass column, 3% OV-17 on Chromosorb W. or Gas Chrom Q.	10% Methane 90% Argon or N ₂	270 ^o C	diazepam	34,50, 51,52
3 ft x 4 mm I.D.; glass coiled. % OV-17 on Gas Chrom. Q	He ₂	225 ^o	tetraphenyl-cyclopentadienone	15

7.095 High Performance Liquid Chromatography

Griseofulvin can be readily chromatographed on either normal or reverse phase columns. Separation of de-chlorogriseofulvin, the most common synthetic impurity is accomplished on C/18, CN (17) or ETH columns. (15) Isogriseofulvin, is separated on either CN (17) or ETH columns (15).

Liquid chromatography conditions are given in Table XIII.

Table XIII

<u>Column</u>	<u>Type</u>	<u>Mobile Phase</u>	<u>Internal Standard</u>	<u>Reference</u>
μBondapak C/18 (octyl-decyl chemically bonded to silica)	Reverse phase	Methanol: water 3:2	n-butyl p-hydroxy benzoate	16
μBondapak C/18 (octyl-decyl chemically bonded to silica)	"	45% aceto-nitrile in 45mM KH_2PO_4 pH = 3.0	diazepan	53
Zorbax CN (cyano-propyl chemically bonded to silica)	"	Methanol: water 3:2	m-phenyl phenol	16
Permaphase ETH (C-7 ether chemically bonded to pellicular 30-50 mesh glass packing)	Normal phase	5% chloroform in hexane		14

7.10 Biological Methods or Analysis

Microbiological procedures have been developed for assay of griseofulvin and applied to the analysis of bulk drugs and dosage forms (1). The cylinder plate agar diffusion method is the official microbiological method of determination (55). *Microsporum gypseum* (ATCC 14683) is the test organism.

8. Identification and Determination in Body Fluids and Tissue

Griseofulvin has usually been determined in body fluids and tissues by spectrofluorimetric (30-34) or gas chromatographic methods (45,46,48). More recently griseofulvin has been determined in plasma by high performance liquid chromatography (53,54).

9. Analysis of Dosage forms

Usual dosage forms of griseofulvin are capsules, tablets and boluses. These may be prepared for analysis by simple liquid solid extraction of drug substance. Margosis (15,50) used chloroform as an extracting solvent with gentle heat. A compendia procedure describes the extraction of griseofulvin from tablets with boiling alcohol. The analysis for extracted drug substance has been performed by several methods. Most common is a simple ultraviolet analysis (2,3,55). Polarography utilizing the system given in Section 7.08 has been used (39). Thin-layer, gas and liquid chromatography may also be

used utilizing systems described in Sections 7.093, 7.094, and 7.095 respectively. These latter methods are valuable because of their specificity.

In the United States, griseofulvin drug substance and dosage forms must conform to the regulations of the Federal Food and Drug Administration concerning antibiotic drugs (55, 56). Microbiological assay results obtained by analytical methods described in these compendia are conclusive.

10. Acknowledgements

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HALCINONIDE

Joel Kirschbaum

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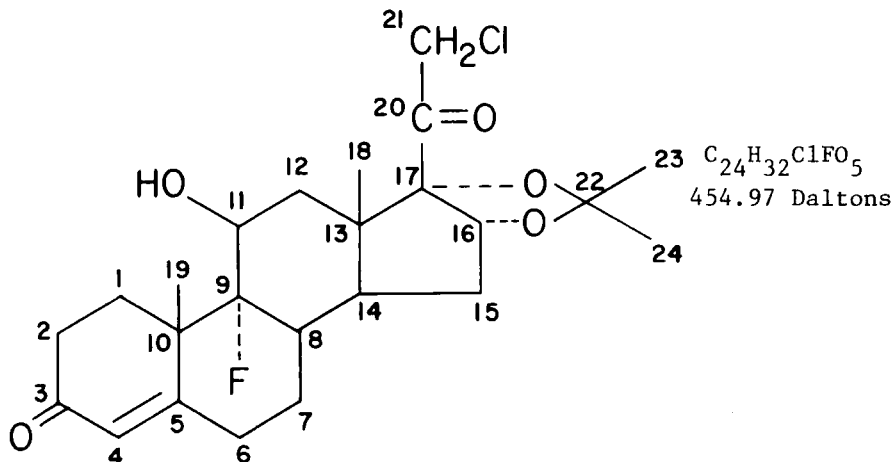
1 History, Description, Precautions and Synthesis

1.1 History

Halcinonide is a topical antiinflammatory agent.^{1,2} Introduction of a 9 α -fluoro group³ to the hydrocortisone molecule resulted in an eight-fold increase in antiinflammatory activity. Such other groups as 16 α -hydroxy, 16 α -methyl, 16 β -methyl, 16,17-acetonide and 6 α -methyl moieties have been found to diminish or eliminate mineralocorticoid activity resulting from the 9 α -fluoro substituent, while retaining the enhanced antiinflammatory activity of the halo derivative⁴. Halcinonide lipophilicity is increased by masking the 16,17-hydroxyl groups as the acetonide, which increases the availability of the steroid at the site of action in the skin. The 21-chloro group also increased antiinflammatory properties. Halcinonide has systemic³ as well as topical activity.

1.2 Names, Formula and Molecular Weight

Halcinonide is the United States adopted name⁵ (USAN). The preferred chemical names⁶ are 21-chloro-9-fluoro-11 β -hydroxy-16 α , 17-[(1-methylethylidene)bis(oxy)]pregn-4-ene-3, 20-dione, and 21-chloro-9 α -fluoro-11 β -16 α ,17-trihydroxypregn-4-ene-3,20-dione cyclic 16,17-acetal with acetone. Other chemical names are 21-chloro-9-fluoro-11 β -hydroxy-16 α ,17 α -isopropylidenedioxy-4-pregnene-3,20-dione, 9 α -fluoro-21-chloro-11 β , 16 α , 17 α -trihydroxypregn-4-ene-3, 20-dione 16,17 acetonide and 21-chloro-9-fluoro-11 β ,16 α ,17-trihydroxypregn-4-ene-3,20-dione cyclic 16,17-acetal with acetone.



Halcinonide has also been called Halog and Squibb SQ 18,566. The Chemical Abstracts systematic number is CAS-3093-35-4.

1.3 Appearance, Color and Odor

Halcinonide is a white or practically white, odorless powder consisting of free flowing crystals.

1.4 Precautions

Since halcinonide is a corticosteroid, large doses of unformulated steroid are needed systemically before the unwanted side effects appear.⁷ Halcinonide is usually formulated at concentrations of 0.1%, or less. Normal handling precautions are adequate.

1.5 Synthesis

The synthesis^{8,9} of halcinonide is summarized in Figure 1, starting with 16 α -hydroxy-9 α -fluorohydrocortisone (Δ^4 -pregnene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione; dihydrotriamcinolone, I), which is available commercially.¹⁰⁻¹³ This tetrahydroxy steroid is slurried in acetone, and then 70% perchloric acid is added slowly. The acetonide, II (9 α -fluoro-11 β ,16 α ,17,21-tetrahydroxypregn-4-ene-3,20-dione, cyclic 16,17-acetal with acetone; dihydrotriamcinolone-acetonide) precipitates spontaneously from solution. Mesyl chloride is added to the acetonide in pyridine to give the 21-mesylate derivative (dihydrotriamcinolone acetonide-21-mesylate, III). Compound III is dissolved in dimethylformamide, lithium chloride is added and the mixture is refluxed to produce halcinonide (IV), which is recrystallized from a solution of *n*-propanol in water.

2. Physical Properties

2.01 Single Crystal X-Ray Diffraction

The three dimensional structure was obtained¹⁴ by means of single crystal X-ray diffraction. CuK α radiation, a graphite monochromator, and a photomultiplier tube were used to collect 1825 total reflections on an automated diffractometer. Of these, 1162 were used for the analysis. Figure 2 shows a computer generated drawing of halcinonide. The position of the chlorine atom was not clear from the Patterson map, but the direct method program "MULTAN" gave its position. Least squares refinement of coordinates together with anisotropic temperature factors, in the final stages, gave an R factor of 0.11.

Figure 1. Synthesis of Halcinonide

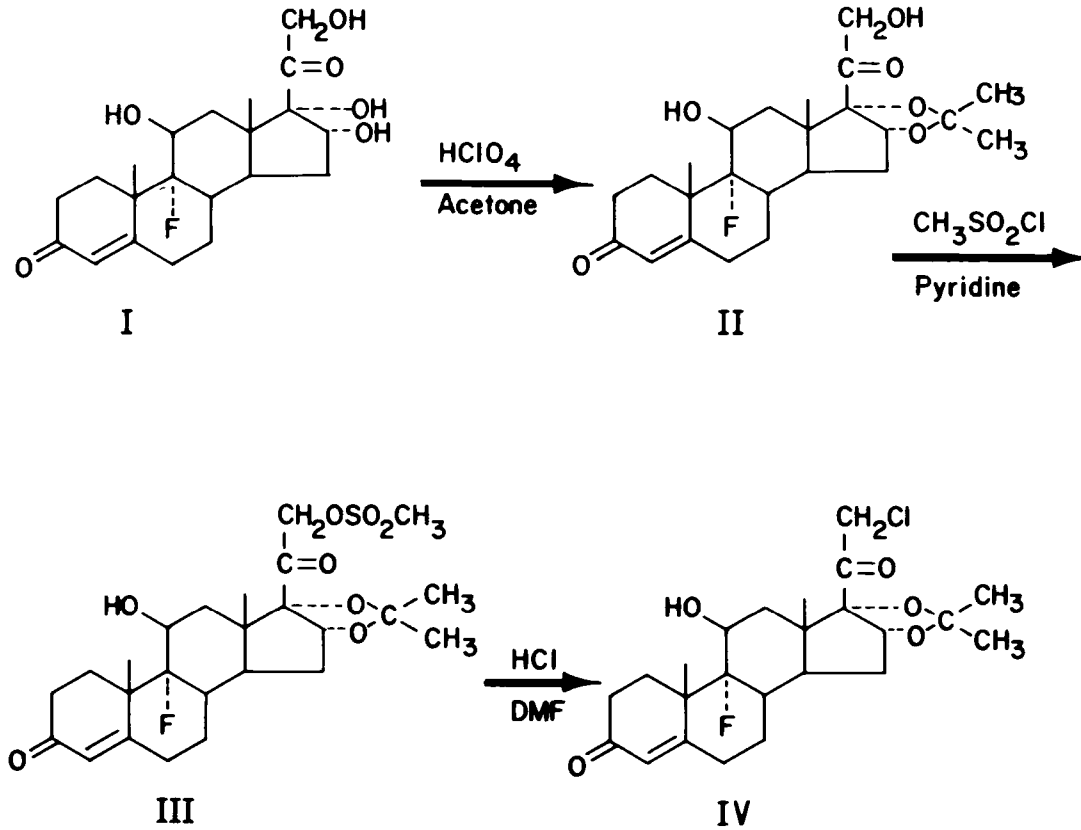
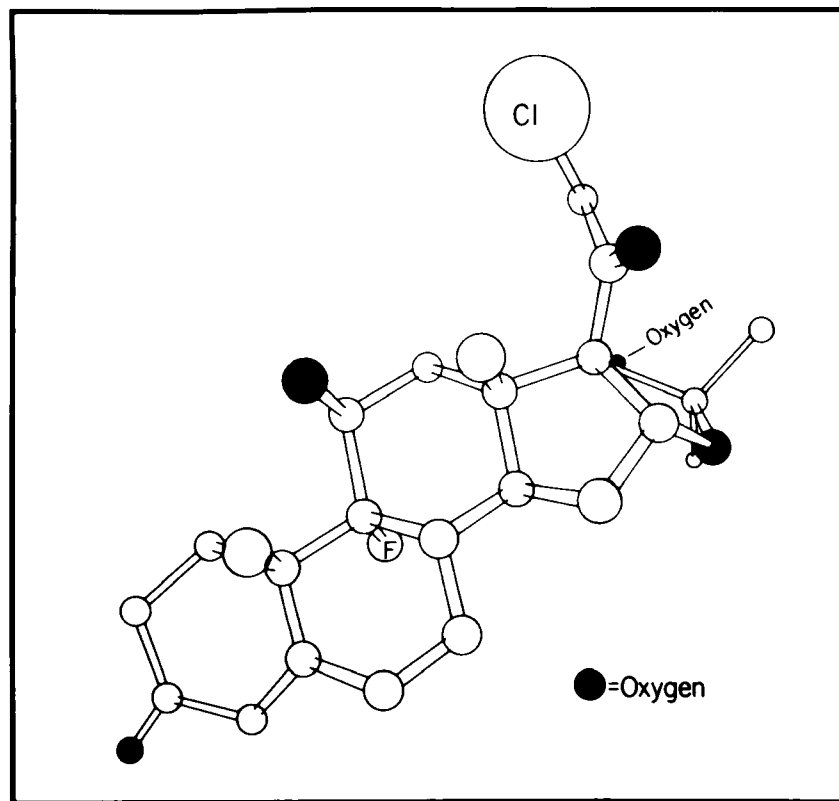


Figure 2. Structure of Halcinonide, from X-ray Diffraction Data.



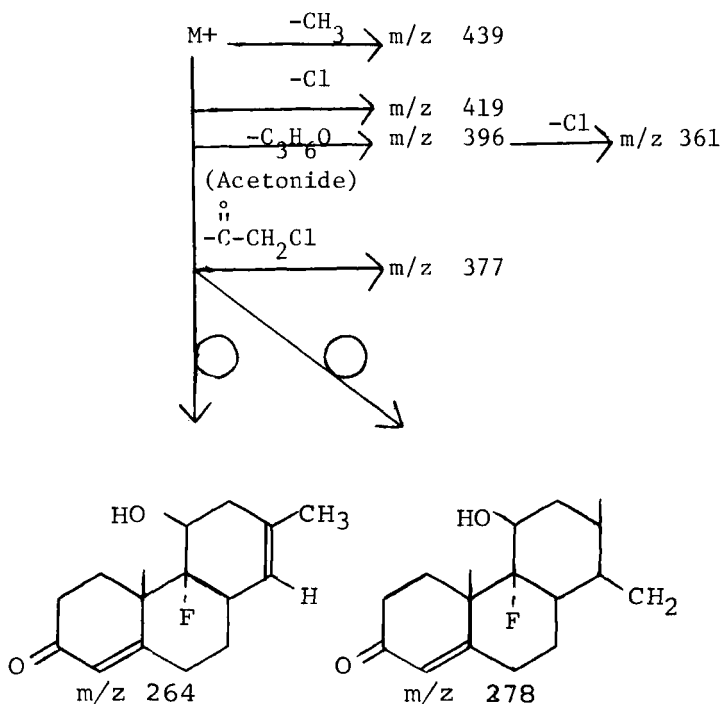
18,566 Computer Generated Drawing

2.02 Mass Spectrometry

Figure 3 is the plotted low resolution mass spectrum of halcinonide found using an AEI Scientific Apparatus Ltd, Model 902 mass spectrometer. Spectra were collected on frequency modulated analog tape and processed on a Digital Equipment Corp., PDP-11.

The table¹⁵ below summarizes high resolution data from m/z 39 to the prominent molecular ion at 454.1922, which corresponds to $C_{24}H_{32}O_5FCl$. Each fragment ion containing chlorine appears as a doublet peak because chlorine is a mixture of two isotopes (^{35}Cl and ^{37}Cl). These peaks are two mass units apart with an intensity ratio of 3 to 1.

Fragmentation proceeds along several pathways, as shown below.

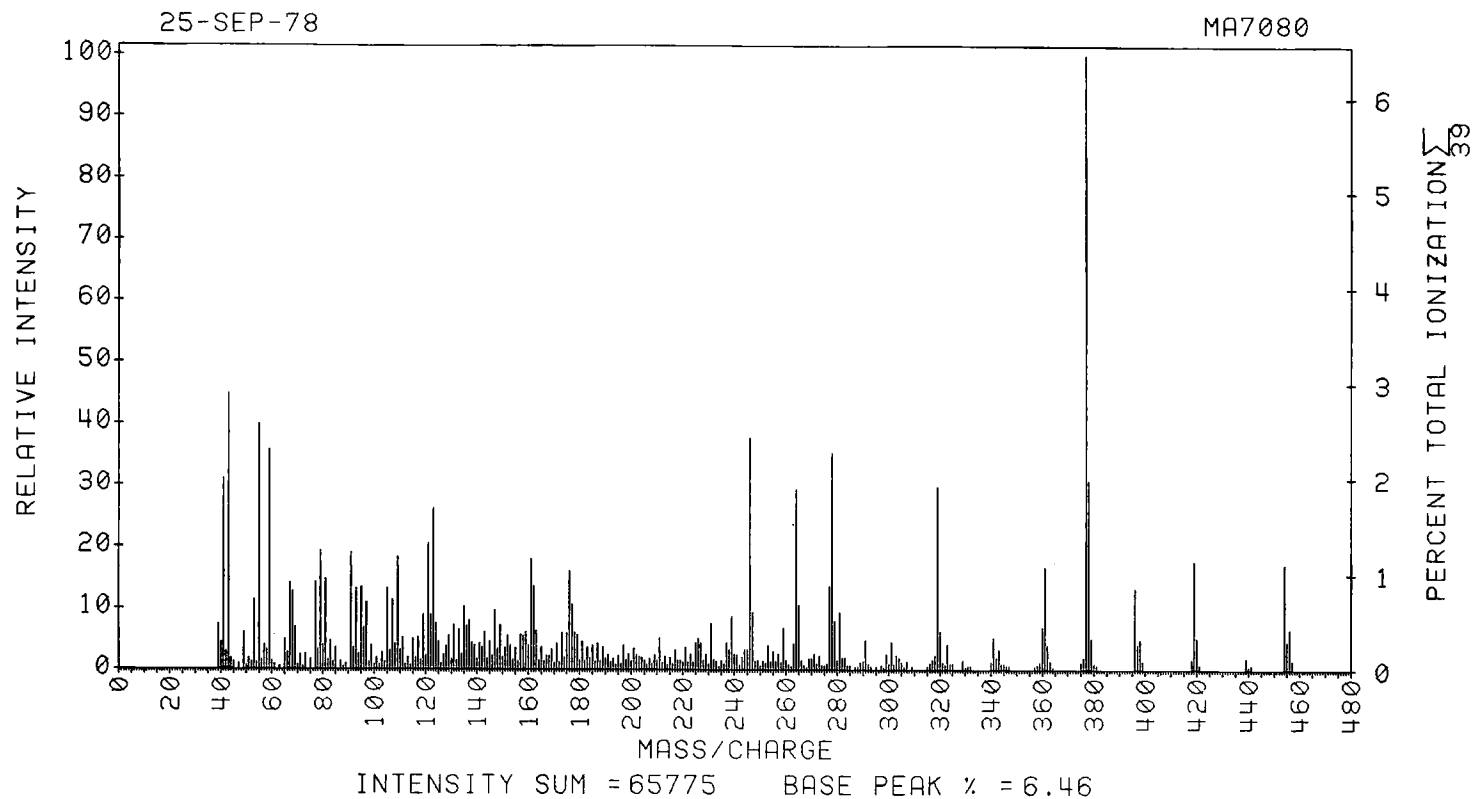


These structures do not necessarily represent the actual ionic structure but only the origin of the fragment.

The weak ions at m/z 121, 122 and 123 (C_8H_9O , $C_8H_{10}O$ and $C_8H_{11}O$) are characteristic of Δ^4 -3-one steroids.

Figure 3

Low Resolution Mass Spectrum of Halcinonide. See text for details.
5433 SQ18,566 BA NN009NA 175 DEG



High-Resolution Mass^a Spectrum of Halcinonide

<u>Found Mass</u>	<u>Calc. Mass</u>	<u>Unsat.^b</u>	<u>O/E^c</u>	<u>C</u>	<u>H</u>	<u>O</u>	<u>F</u>	<u>Cl</u>
454.1925	454.1922	8.0	O	24	32	5	1	1
439.1680	439.1687	8.5	E	23	29	5	1	1
419.2252	419.2233	8.5	E	24	32	5	1	0
396.1501	396.1503	8.0	O	21	26	4	1	1
377.2147	377.2128	7.5	E	22	30	4	1	0
361.1838	361.1815	8.5	E	21	26	4	1	0
319.1665	319.1709	7.5	E	19	24	3	1	0
278.1695	278.1682	6.0	O	17	23	2	1	0
264.1524	264.1525	6.0	O	16	21	2	1	0
246.1422	246.1420	7.0	O	16	19	1	1	0
135.0808	135.0810	4.5	E	9	11	1	0	0
123.0769	123.0810	3.5	E	8	11	1	0	0
122.0729	122.0732	4.0	O	8	10	1	0	0
121.0683	121.0653	4.5	E	8	9	1	0	0
76.9790	76.9794	1.5	E	2	2	1	0	1
43.0184	43.0184	1.5	E	2	3	1	0	0

^aOnly those peaks considered to be significant to the discussion are listed. A complete element map can be obtained on request.

^bNumber of double bonds and rings.

^cO-odd electron ion; E-even electron ion.

2.03 Nuclear Magnetic Resonance Spectrometry (NMR)2.031 ^1H -NMR

Figure 4 is the 100 MHz NMR Spectrum of halcinonide in deuteriochloroform containing tetramethylsilane as internal reference at 0 Hz. The instrument used is a Varian Associates, Inc., Model XL-100 NMR spectrometer. Below is an interpretation of the various resonances.¹⁶

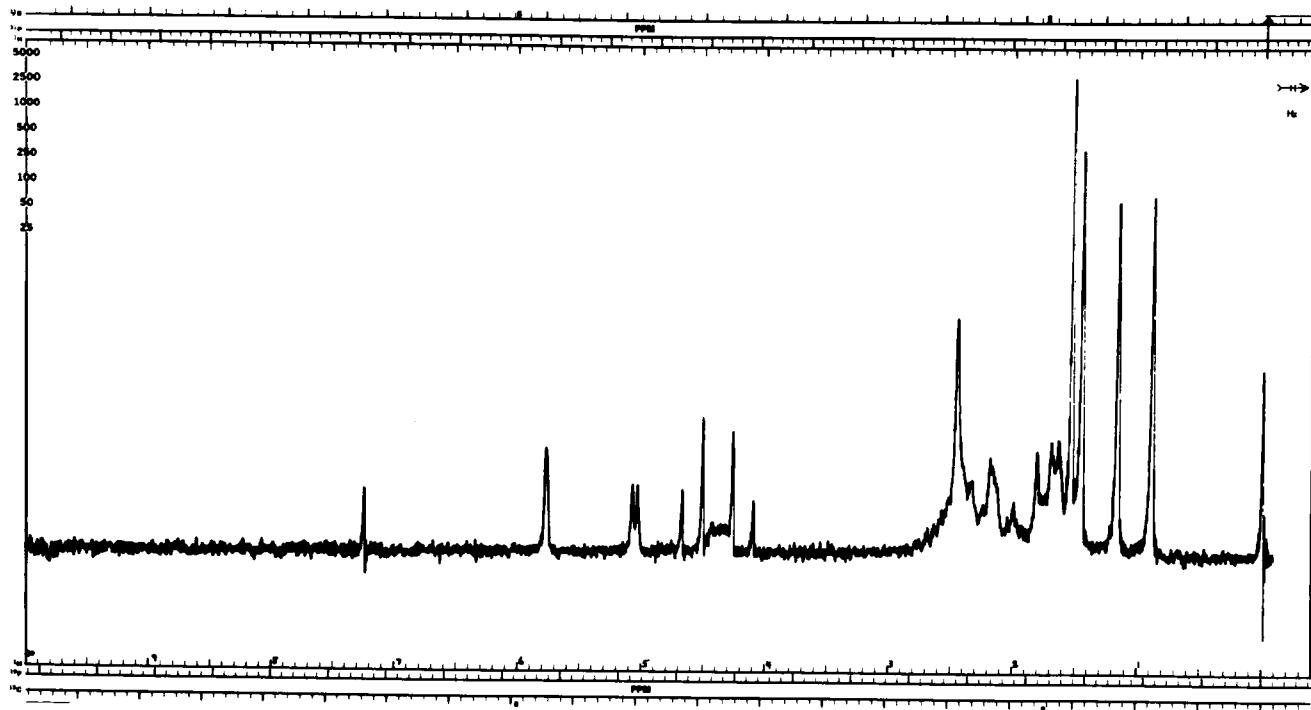
 ^1H -NMR Assignments

<u>Proton at Carbon</u>	<u>Chemical Shift</u>	Peak Appearance
		<u>S = singlet</u> <u>D = doublet</u> <u>B = broad</u>
C-4	5.78	S
C-11	4.58	B
C-16	5.05	D $J_{15,16} = 4.0$ Hz
C-18	0.87	S
C-19	1.51	S
C-21	4.19 4.57	AB quartet
β -Acetonide, methyl	1.15	S
α -Acetonide, methyl	1.44	S

These data agree with the published results of the 21-hydroxy- Δ^1 analog, triamcinolone acetonide.¹⁷

Figure 4

^1H -Nuclear Magnetic Resonance Spectrum of Halcinonide
See text for details.



2.032 ^{13}C -NMR

The ^{13}C -NMR spectrum of halcinonide, Figure 5, was obtained¹⁸ using a Jeol FX-60Q NMR spectrometer operating in the Fourier transform mode at 15 MHz. Numbers on the various peaks refer to the assignments in the table below. These assignments were made by comparison of chemical shifts and ^{13}C - ^{19}F coupling constants to those assigned to such related steroids as 9 α -fluorocortisol (9 α -fluoro-11 β ,17,21-trihydroxy-pregn-4-ene-3,20-dione).

Four pairs of closely related peaks show chemical shift differences small enough to lead to possible reversals of their tentative assignments. These pairs are the peaks assigned to the acetone methyl groups ($\delta = 28.6$ and $\delta = 25.1$ ppm), the two carbonyl peaks ($\delta = 202.1$ and 199.1 ppm), the methylene carbons assigned to C-1 and C-7 ($\delta = 26.2$ and 28.5 ppm), and the methylene carbons assigned to C-2 and C-15 ($\delta = 33.2$ and 33.7 ppm). These assignments must be verified by additional experimentation.

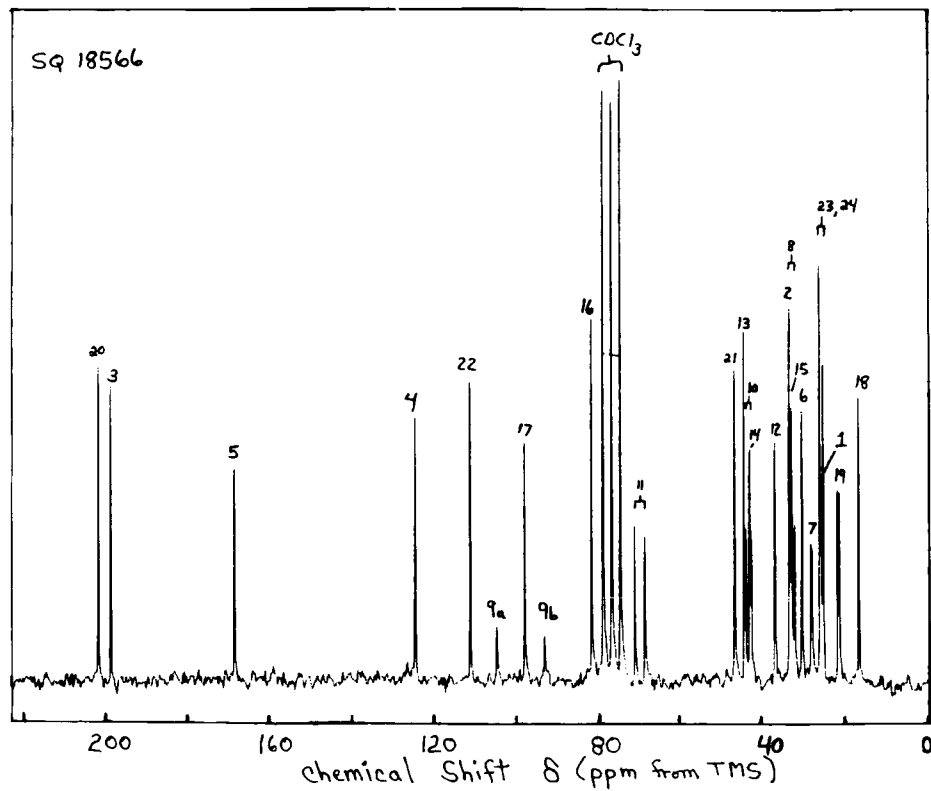
 ^{13}C -NMR Assignments

Carbon	Chemical Shift δ (ppm) ^a	C-F Coupling Constant (Hz)
1	26.2 (28.5)	3
2	33.7 (33.2)	
3	199.1 (202.1)	
4	124.7	
5	169.0	
6	30.7	3
7	28.5 (26.2)	
8	33.1	
9	99.0	
10	43.7	
11	70.2	2
12	37.3	
13	44.8	
14	43.4	
15	33.2 (33.7)	
16	81.9	6
17	98.1	
18	17.0	
19	21.9	
20	202.1 (199.1)	
21	47.1	
22	111.5	
23	28.6 (25.1)	
24	25.1 (28.6)	

^aReferenced from center peak of deuteriochloroform = 77.0 ppm from tetramethyl silane. Numbers in parentheses refer to alternative assignments (see text for details).

Figure 5

¹³C-Nuclear Magnetic Resonance Spectrum of Halcinonide
See text for details.



2.04 Infrared Spectrometry

Figure 6 shows the infrared spectrum of halcinonide run as a potassium bromide pellet, using a Perkin-Elmer Model 621 infrared spectrometer. Below are the interpretations of various absorbances.¹⁹

<u>Frequency (cm¹)</u>	<u>Interpretation</u>
3450	-OH Stretch
2950	-OH Stretch
1730	C ₂₀ Keto
1660	C ₃ Keto
1620	Δ ⁴ C=C

2.05 X-ray Powder Diffraction

Figure 7 is the powder X-ray diffraction pattern of halcinonide as obtained on a Philips powder diffraction unit emitting CuKα radiation at 1.54Å. Using a scintillation counter detector, the sample was scanned and recorded from approximately 2 to 40 degrees (2θ). The table below is the sorted data.²⁰

<u>2θ (Degrees)</u>	<u>'d'(Angstrom)¹</u>	<u>Relative Area²</u>
14.80	5.99	1.000
12.53	7.06	0.866
18.49	4.80	0.738
17.94	4.94	0.526
19.82	4.48	0.422
29.31	3.05	0.266
18.10	4.90	0.246
11.67	7.58	0.225
32.14	2.78	0.195
39.59	2.28	0.171
25.86	3.45	0.165
21.47	4.14	0.142
26.80	3.33	0.135
25.24	3.53	0.135
15.67	5.66	0.131
40.37	2.23	0.124
22.33	3.98	0.117
33.08	2.71	0.114
25.47	3.50	0.053
34.02	2.64	0.052

¹Interplanar distance

²Relative area, or intensity, is based on highest intensity of 1.00 using CuKα radiation.

Figure 6
Infrared Spectrum of Halcinonide, Potassium Bromide Pellet.
See text for details.

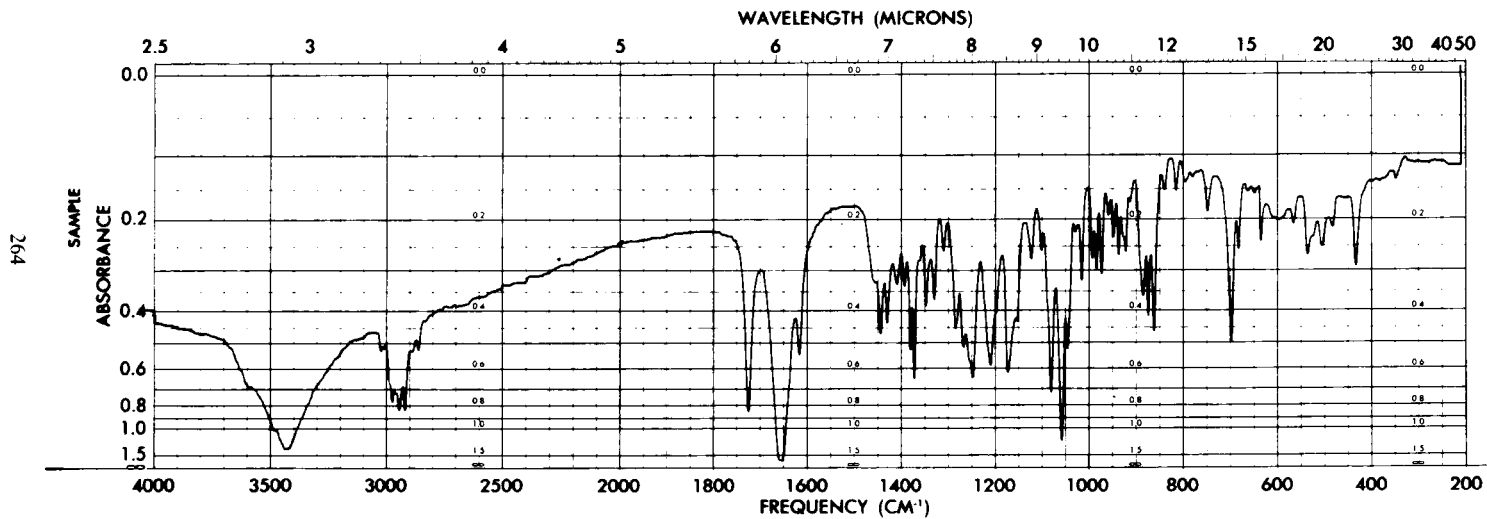
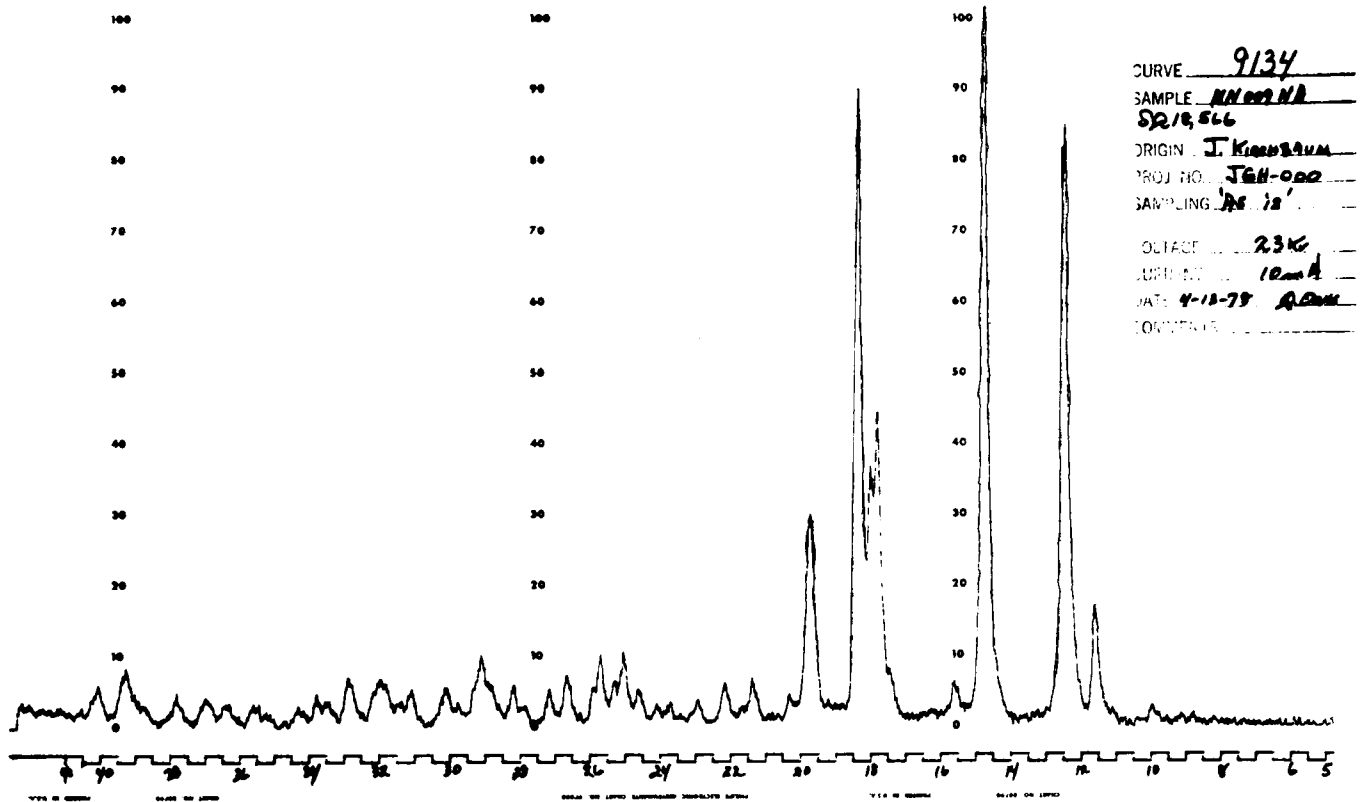


Figure 7

Powder X-ray Diffraction Pattern of Halcinonide. See text for details



2.06 Ultraviolet Spectrometry

Ultraviolet spectra²¹ of halcinonide were recorded on a Beckman Acta CIII spectrophotometer. The spectra in methanol, 0.1M methanolic hydrochloric acid and 0.1M methanolic sodium hydroxide show peak maxima at 238 nm, and remain unchanged after 24 hours. Absorbances are tabulated below using traditional $E_{1\text{cm}}^{1\%}$ notation.

<u>Solvent</u>	$E_{1\text{cm}}^{1\%}$		
	<u>Initial</u>	<u>3 Hours</u>	<u>24 Hours</u>
Methanol	379	374	373
0.1M Methanolic	370	372	370
Hydrochloric Acid			
0.1M Methanolic	380	382	380
Sodium Hydroxide			

The spectrum in 95% ethanol also has a peak maximum at 238 nm and appears stable for at least 3 hours. In acetonitrile and ethyl ether the maximum is shifted to 234 nm and 229 nm, respectively.

2.07 Optical Rotation

The specific rotations in alcohol²² were determined²³ to be as follows,

<u>Wavelength (nm)</u>	<u>Specific Rotation</u>
589	+156
578	+164
546	+188
436	+348
305	+436

2.08 Fluorescence Spectrometry

Using a Perkin-Elmer Model 204 fluorescence spectrophotometer, no fluorescence²⁴ was found at either 1 or 1000 μg halcinonide per mL of methanol. No fluorescence was induced in 0.1M hydrochloric acid or 0.1M sodium hydroxide.

2.09 Melting Range

Following the USP procedure²⁵ for class 1A compounds, the melting range of halcinonide is 270-272° (reference 21). This is in excellent agreement with the results of differential thermal analysis, below, as well as hot stage microscopy, section 2.12.

2.10 Differential Thermal Analysis

A duPont Model 900 Differential Thermal Analyzer shows halcinonide to have one endotherm²⁶ at 269°. Decomposition on melting precludes differential scanning calorimetry studies for purity.

2.11 Thermal Gravimetric Analysis

Using a Perkin-Elmer Model TGS-2 Thermogravimetric Analyzer, halcinonide was found²⁶ to lose 0.3% total volatile material at 70°. No further loss was found up to 150°. The heating rate was 20°/minute under a nitrogen atmosphere.

2.12 Microscopy and Crystal Type

Microscopically, the crystal type depends on the method of preparation of halcinonide. Slabs, either 2 to 4, 5 to 10, or 10 to 15 microns square, were found in three lots of halcinonide, intermingled with needle-like (acicular) crystals 5 to 10 microns long.²⁷

Single crystal x-ray diffraction studies¹⁴ showed that the crystals of halcinonide recrystallized from *n*-propyl alcohol-water azeotrope (79:22) are orthorhombic and belong to the space group $P2_12_12_1$, with unit cell constants of $a = 10.007$ Å, $b = 11.875$ Å and $c = 19.460$ Å. Density is 1.330 gm/cm³, as measured by flotation in a hexane-carbon tetrachloride gradient. The molecular weight calculated from the unit cell volume and density is 461 daltons (theoretical is 455 daltons).

Hot stage microscopy²⁶ was performed using a Mettler FP52 temperature controller at a rate of 3°/minute. Melting began at 268.2°, and by 271.4° all crystals were melted. The yellow melt slowly turned orange-brown. Cooling showed no recrystallization at ambient temperature.

2.13 Particle Size

By light scattering using a Royco instrument, all particles are below 20 microns.²⁷ Coulter Counter analysis of ground halcinonide suspended in a sodium chloride solution shows 100% to be $<10.2\mu$ (μ = micron), 98.2% $<6.4\mu$, 95.3% $<5.1\mu$, 52.0% $<2.6\mu$ and 19.9% $<1.6\mu$.

2.14 Surface Area

As measured by gas adsorption,²⁷ the surface areas of three lots of ground halcinonide are 2.61, 3.85 and $4.08 \text{ m}^2/\text{g}$.

2.15 Polymorphism

There is no evidence for polymorphism from infrared spectroscopy and differential thermal analysis, and only inconclusive data from x-ray diffraction and microscopy studies.

2.16 Hydration

The crystals of halcinonide are not solvated with water, based on a total volatile content of 0.3% obtained by thermal gravimetric analysis, a correct elemental analysis, and a loss-on-drying value of 0.6% (cf. section 4.1, Elemental and Inorganic Analyses).

3. Solution Properties

3.1 Intrinsic Dissolution Rate

The intrinsic dissolution rate was determined after compressing powder under 2000 p.s.i.g. pressure using 3/8" diameter disc-shaped dies. In one liter of 0.1M hydrochloric acid at 37°, agitated at a rate of 50 r.p.m., the intrinsic dissolution rate of halcinonide is $8.33 \times 10^{-3} \text{ mg min}^{-1} \text{ cm}^{-2}$ computed from data obtained by ultraviolet spectrometry.²⁸

3.2 Solubilities in Aqueous and Nonaqueous Solvents

Solubilities of halcinonide were determined in various solvents.²⁹ Results are reported using the U.S.P. definitions.³⁰

Solvent	Solubility
Water	Insoluble
Hydrochloric acid, 0.1 <i>M</i>	Insoluble
Sodium hydroxide, 0.1 <i>M</i>	Insoluble
Acetone	Soluble
Acetonitrile	Sparingly soluble
Acetonitrile-water	Slightly soluble
Benzene	Slightly soluble
Chloroform	Freely soluble
Castor oil	Slightly soluble
Dimethylsulfoxide	Freely soluble
Ethanol	Slightly soluble
Ethyl ether	Slightly soluble
Glyceryl monooleate	Slightly soluble
Hexanes	Insoluble
Isopropyl myristate	Slightly soluble
Methanol	Slightly soluble
<i>n</i> -Octanol	Slightly soluble
Polyethylene glycol 200	Slightly soluble
Polyethylene glycol 400	Slightly soluble
Polypropylene glycol	Slightly soluble
Propylene glycol	Very slightly soluble

3.3 Partition Coefficients

Halcinonide was partitioned³¹ between hexanes and methanol, and between hexanes and aqueous acetonitrile at apparent pH values of 2,4,6(unadjusted), and 10. After one hour of mixing, the steroid content was determined by ultra-violet spectrometry of both phases. In all cases, absorbance at the peak maximum of 239 nm was detected only in the acetonitrile-water or methanol layer. The aqueous acetonitrile (pH 6) result was verified³² using ¹⁴C-halcinonide labeled at the 2- carbon of the acetone moiety. Thus, the halcinonide is completely retained in either the acetonitrile-water or methanol layers, indicating the utility of these solvent systems for extracting the steroid from formulations.

Another study³³ involved isopropyl myristate and aqueous propylene glycol. After equilibration for two weeks at 37°, the layers were separated³⁴ and the aqueous glycol assayed for steroid using high-pressure liquid chromatography (*cf.* section 4.31). The isopropyl myristate/aqueous propylene glycol partition coefficients are as follows: propylene glycol-water (4:1); 144; propylene glycol-water (2:3), 44.4; propylene glycol-water (3:2), 8.14, and propylene glycol-water (9:1), 0.82.

4. Methods of Analysis

4.1 Elemental and Inorganic Analyses

The elemental analysis³⁵ of halcinonide is carbon 63.38% (63.35%, theoretical); hydrogen 7.39% (7.10%, theoretical); chlorine, 7.89 (7.79%, theoretical), and fluorine, 4.30% (4.17%, theoretical).

Emission spectrochemical analysis for metals was performed using a Spec Industries carbon arc A.C. unit with a Bausch and Lomb dual grating spectrograph. Data were recorded on glass plates and interpreted by means of a microphotometer. Halcinonide contained trace amounts (~ 60 µg/g total) of the following metallic impurities³⁶; iron, manganese, copper, nickel, lead, zinc, aluminum, sodium, calcium, and magnesium. Residue-on-ignition³⁷ of the same lot³⁸ is less than 0.1%. Heavy metals content (reference 39, method II) is less than 0.003%.³⁸ After 3 hours at 100° in vacuum, the loss-on-drying³⁹ value³⁸ is 0.6%.

Four lots of halcinonide were analyzed for residual solvents by dissolving portions in pyridine. After retention on a precolumn, water content was determined by

vapor phase (gas) chromatography using an authentic standard before and after each injection. Water contents of 0.6%, or less, were found by comparison with authentic standards. *n*-Propanol has occasionally been found to be present.

4.2 Identification, Ultraviolet and Colorimetric Analyses

Proposed compendial identification tests for the *United States Pharmacopeia* involve comparing either the infrared or ultraviolet absorption spectrum of sample halcinonide with that of an authentic sample⁴¹. To this author, a chromatographic method is superior since elution time usually depends on much of the molecule interacting with the solid and mobile phases via weak bonding forces. Ultraviolet absorption depends principally on the 3-one-4-ene, A and B ring region being intact.

Halcinonide has been quantitated in various formulations or as bulk powder by a differential ultraviolet, borohydride reduction assay.⁴² This differential assay involves measuring the ultraviolet absorbance of an aliquot of methanolic steroid solution containing sodium borohydride decomposed prior to the addition of steroid. Its absorbance is determined against a methanolic reference solution of steroid reduced by sodium borohydride to destroy the 3-one-4-ene chromophore. The utility of this procedure is that many interferences from excipients and other, unconjugated, steroids can be eliminated in the assay of a formulation.

The addition of halcinonide to various colorimetric reagents gives results typical of steroids with its substituents.⁴³⁻⁴⁵ As tested in our laboratory,⁴⁶ halcinonide reacts with acidic ethanolic 4-nitrophenylhydrazine⁴⁷, after heating, cooling and the addition of sodium hydroxide, to give a brilliant purple "plum" color. With methanolic isoniazid⁴⁸, halcinonide gives a bright yellow color, with no noticeable fading after two hours. Halcinonide added to 4-aminoantipyrine⁴⁹ in methanolic hydrochloric acid gives a pale green color. Halcinonide added to ethanolic tetramethylammonium hydroxide⁵⁰, and heated, gives a cloudy amber color. This can be the basis of a quantitative assay.⁵¹ If added to ethanolic tetramethylammonium hydroxide⁵² and picric acid, an orange-red (tea colored) solution results with halcinonide. In concentrated sulfuric acid,⁵³ halcinonide gives a deep yellow color. Adding water slowly causes a violet color to appear at the interface. Halcinonide gives a royal purple color with blue tetrazolium⁵⁴ and a yellow-brown color with aluminum chloride in nitromethane.

4.3 Chromatographic Analyses

4.31 High-Pressure Liquid Chromatography (HPLC)

Reverse phase high-pressure liquid chromatography is used to separate and quantitate bulk and formulated halcinonide.⁵⁵ Commercially available, prepacked octadecylsilane columns, 10 μm in particle size, *United States Pharmacopeia* designation L-1 (such as Partisil, MicroPak or μ Bondapak), are used. The mobile phase is aqueous acetonitrile (1:1 to 1:3) with a flow rate of 0.3 to 1.0 mL/min. Detection is at 254 nm. Figure 7 shows the elution of halcinonide and progesterone internal standard. Using a precision loop injector, repetitive injections gave a relative standard deviation of 0.6%. Without the progesterone internal standard, the relative standard deviation is 0.8%. Formulated halcinonide, in concentrations of 1 to 0.25 mg steroid/g, is either diluted with mobile phase, extracted into methanol, or partitioned into aqueous acetonitrile from hexanes^{55,56}. Halcinonide can be separated from kenalog¹⁷ (triamcinolone acetate) by HPLC.⁵⁷

Intermediates in the synthesis of halcinonide (*cf.* section 1.5) have the following relative retention times; Dihydrotriamcinolone, $R_{\text{RT}} = 0.18$; dihydrotriamcinolone acetate, $R_{\text{RT}} = 0.32$, and dihydrotriamcinolone acetate-21-mesylate, $R_{\text{RT}} = 0.72$ (Halcinonide, $R_{\text{RT}} = 1.00$)⁴⁶. The purity of dihydrotriamcinolone can be determined by HPLC using the same reverse phase column and a mobile phase consisting of 0.8% ammonium nitrate, 0.23% monobasic ammonium phosphate, 32.5% methanol, 8% tetrahydrofuran and water.⁵⁸ Cortisone acetate is used as an internal standard.⁵⁹ Impurities are determined at a higher concentration.

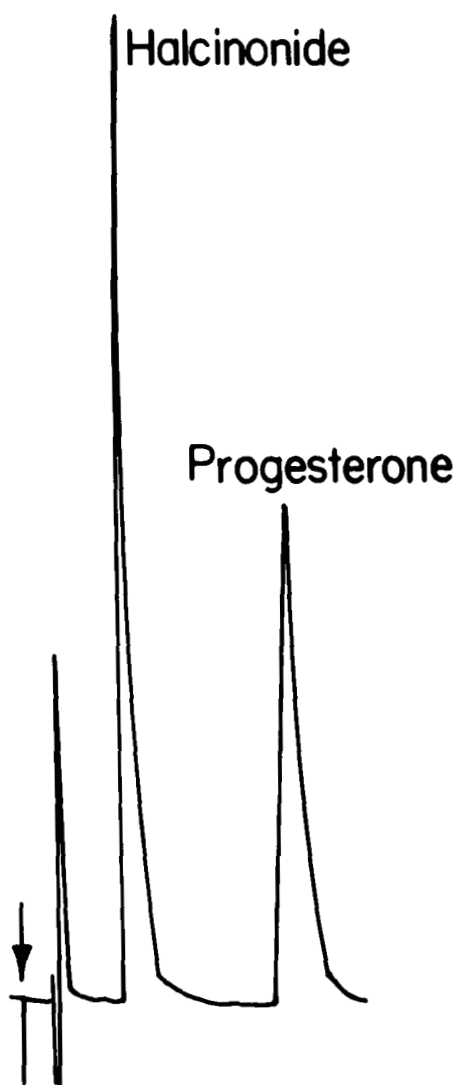


Figure 8

Reverse Phase High-Pressure Liquid Chromatography of Halcinonide. Progesterone is used as internal standard. See text for details.

4.32 Thin Layer Chromatography (TLC)

TLC, using silica gel GF 254 plates, can separate halcinonide from its synthetic precursors (*cf.* section 1.5). Using a developing solvent of chloroform-ethyl acetate (5:1), the following R_f values were found⁶⁰: Dihydrotriamcinolone, R_f = 0.0; dihydrotriamcinolone acetate, R_f = 0.08; dihydrotriamcinolone acetate-21-mesylate, R_f = 0.18 and halcinonide, R_f = 0.36. Continuous TLC development gives respective relative R_f values of 0.02, 0.02, 0.48 and 1.0. With benzene (CAUTION)-acetone-water (70:30:07) the respective R_f values are 0.12, 0.42, 0.66 and 0.81. Traces of these intermediates have been found in some lots of halcinonide, as has dihydrotriamcinolone-21-acetate, an impurity occasionally found in some preparations of dihydrotriamcinolone. Elution from the TLC plate with ethanol permits quantitation of bulk halcinonide and halcinonide extracted from formulations. ¹⁴C-Halcinonide (labelled at the acetate carbon) was examined for impurities using silica gel TLC plates and chloroform-ethyl acetate (5:1)⁶¹ mobile phase. The R_f value of halcinonide is 0.5.

4.33 Column Chromatography

A diatomaceous earth column was used to separate halcinonide from excipients⁶². The formulation is mixed with column packing material and then is transferred to the top of the column. Steroidal material is eluted with benzene (CAUTION) under a well-ventilated hood and then quantitated using thin layer chromatography⁶⁰ or tetramethylammonium hydroxide⁵¹ colorimetry.

4.34 Paper Chromatography

Paper chromatography using Whatman No. 1 paper was once used to determine the homogeneity of halcinonide.⁶⁰ Twenty percent formamide in methanol comprises one stationary phase and methylisobutyl ketone-formamide (20:1) is the mobile phase. A second solvent system uses 25% propylene glycol-chloroform as the stationary phase and toluene saturated with propylene glycol as the mobile phase.

4.4 Polarographic Analysis

In dimethylformamide, halcinonide is reduced in two steps.⁶³ The 21 α -chloroketo group exhibits a half-wave reduction potential of -1.17 volts *vs* Hg. This is easily distinguished from the half-wave potential of -1.62 volts *vs* Hg of the Δ^4 -3-keto group. Thus, halcinonide can be

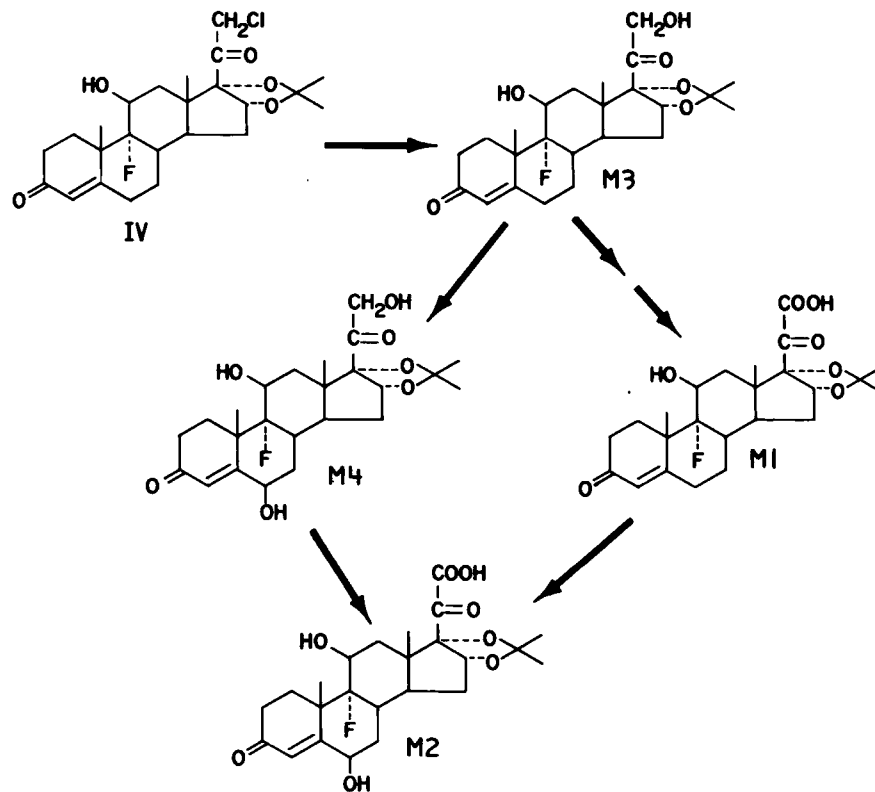
assayed in the presence of other 4-ene-3-keto steroids that lack the 21-chloro group. This response requires a concentration between 0.5 and 2.0 mM of steroid per liter. The more sensitive technique of differential pulse polarography⁶⁴ should also be applicable to halcinonide.

4.5 Halcinonide in Tissues and Body Fluids

The antiinflammatory properties of such topical agents as halcinonide are usually determined by a vasoconstrictor assay. Topically applied corticosteroids cause a blanching at the site of application, which can be the forearm⁶⁵ or the upper back of healthy adults where stratum corneum is removed with cellophane tape.⁶⁶ The test areas, containing various concentrations of halcinonide, are occluded with plastic wrap and are evaluated on an all-or-none basis.³ Percutaneous absorption studies⁶⁷ with 0.1% ¹⁴C-halcinonide cream, 1 g cream per dog or rabbit, showed that the steroid is absorbed through intact or abraded skin. In dogs, 0.4 to 0.5% is estimated to be absorbed through intact and 4 to 10% through abraded skin. In rabbits, 6 to 16% of the ¹⁴C-halcinonide was absorbed through intact and 14 to 23% through abraded skin.

Metabolism⁶⁸ was studied with halcinonide labelled with carbon-14 in the 2-position of the acetone group. It was administered intravenously to dogs at a dose of 5 mg/kg. The major portion of the radioactivity was excreted in bile. Radio-autography of bile showed at least 10 distinct metabolites to be present. Four of the metabolites were identified. The two most abundant metabolites, that were identified, accounted for 43% (Figure 8, M1) and 30%(M2) of the radioactivity. The two minor metabolites (M3 and M4) accounted for 2% each. In dog urine, these four metabolites (M1-4) accounted for 10, 15, 5 and 18% of the radioactivity, respectively. In dog blood, unchanged halcinonide and metabolites M3 and M4 each accounted for about 15% of the radioactivity. M1 and M2 were not detected. These four metabolites, none of which contained chlorine, were identified by comparing their ultraviolet, nuclear magnetic resonance or mass spectra, and their thin-layer chromatographic behavior (either underivatized, or as a suitable derivative) with similar data obtained for authentic reference samples. M1 was identified as the 21-carboxy derivative of halcinonide ($-\text{CH}_2\text{Cl} \rightarrow -\text{CO}_2\text{H}$), M2 is the 6 β -hydroxy derivative, M3 is the 21-hydroxy derivative ($-\text{CH}_2\text{Cl} \rightarrow -\text{CH}_2\text{OH}$) and M4 is the 6 β -hydroxy derivative of M3. Figure 9 shows the proposed pathway. M3 is the acetone deriva-

Figure 9
Proposed Metabolic Pathways for Halcinonide in the Dog.



tive in the synthesis of halcinonide (*cf.* Fig. 2) and it is also found as a degradation product in *in vitro* studies summarized below. This compound, dihydrotriamcinolone acetone, possesses topical and systemic antiinflammatory activity.⁶⁹

5. Stability:

Halcinonide dissolved in either methanol, deuterio-methanol, aqueous ammonia-methanol, or deuterium oxide deuterated ammonia-methanol solvents appears stable after storage for six days at 50°, using nuclear magnetic resonance and mass spectrometry.⁷⁰

Using thin layer chromatography (*cf.* section 4.32, first system)⁶⁰ no change was found in two lots of halcinonide after storage at 50° for six months in brown glass bottles. But the direct exposure to 900 foot-candles of light for one month caused an increase in impurities of two lots from 0.5% to 3.9% or 4.9%. Photolytic degradation of the A-ring is expected since hydrocortisone and prednisolone undergo rearrangements when solutions of these steroids in alcohol are exposed to ultraviolet radiation or ordinary fluorescent lighting.⁷¹⁻⁷³

Formulated halcinonide, at a concentration of 0.1% in either a cream base or polyethylene glycol-water lotion, after storage at approximately 23° for 3 years, showed no loss on halcinonide content, using high pressure liquid chromatography.⁴⁶ The contents remained unchanged within 2% of labeled concentration.

One trial formulation, stored at 60° for 7 months showed degradation.⁷⁴ The one impurity that was isolated and identified (with the aid of mass spectrometry) is dihydrotriamcinolone acetone (Figure 1), an intermediate in the synthesis of halcinonide. This compound also possesses topical antiinflammatory activity.⁶⁹

6. Acknowledgements

The author gratefully acknowledges the courteous assistance of the many contributors cited as "personal communication." Some of the information cited in this manner was especially obtained for this analytical profile. Unless otherwise identified, the work was done at E. R. Squibb & Sons, Inc. Much credit for this profile belongs to Dr. Klaus Florey, who wrote the original one for use at Squibb. Special thanks go to R. Poet, G. Brewer, K. Florey and S. Perlman for their critical reading of this MS, to C. Pope for typing it and to my family, for their patience during the compiling of this analytical profile.

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Literature reviewed to January 1, 1979.

HYDRALAZINE HYDROCHLORIDE

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Daley*

1. Description
 - 1.1 Name, Formula, Molecular Weight
 - 1.2 Appearance, Color, Odor
 - 1.3 History
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 - 2.2 Ultraviolet Spectra
 - 2.3 Mass Spectrum
 - 2.4 Nuclear Magnetic Resonance Spectra
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 - 6.8 Paper Chromatography
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 - 6.10 High Pressure Liquid Chromatography
 - 6.11 Gas Chromatography
7. Acknowledgments
8. References

was scanned from 200 to 530 cm^{-1} . Some of the absorption bands can be assigned as follows:

<u>Frequency, cm^{-1}</u>	<u>Assignment (5)</u>
3220	N-H stretch
3025	Aromatic C-H stretch
2800-3000	Mineral oil C-H stretch
1590-1600	C=C stretch
1460	Mineral oil C-H band
785	Out of plane bending, 4 adjacent H atoms on an aromatic ring

The mineral oil absorption at 2800 to 3000 cm^{-1} and at 1460 cm^{-1} obscures absorption bands of hydralazine hydrochloride at 2810, 2920, and 2970 cm^{-1} (N-H^+ stretch) and a weak sharp band at 1470 cm^{-1} ; these bands can be observed in potassium bromide dispersion spectra. The bands at 1070 and 1082 cm^{-1} tend to merge into a single band in potassium bromide dispersion spectra.

The infrared spectrum of hydralazine hydrochloride base in a potassium bromide dispersion (Figure 2) was recorded from 400 to 4000 cm^{-1} , and the 200 to 550 cm^{-1} region was obtained from a mineral oil dispersion supported on polyethylene film. The spectra of potassium bromide dispersions of the base are qualitatively identical to those of mineral oil dispersions. The assignment of absorption bands in the spectrum of the base is similar to that of the hydrochloride except for the presence of N-H^+ stretch absorption in the latter. A spectrum of the base has been published (6).

2.2 Ultraviolet Spectra

Figure 3 is the ultraviolet absorption spectrum of hydralazine hydrochloride in water solution, run on a Cary Model 14 spectrophotometer. The solution contained 9.9 mg of hydralazine hydrochloride per liter, and was run against water (1 cm cells). The discontinuity in the spectrum at 219 nm is a change of absorption scale; the absorbance scale range is 0.0 to 1.0 for the wavelength range 350 to

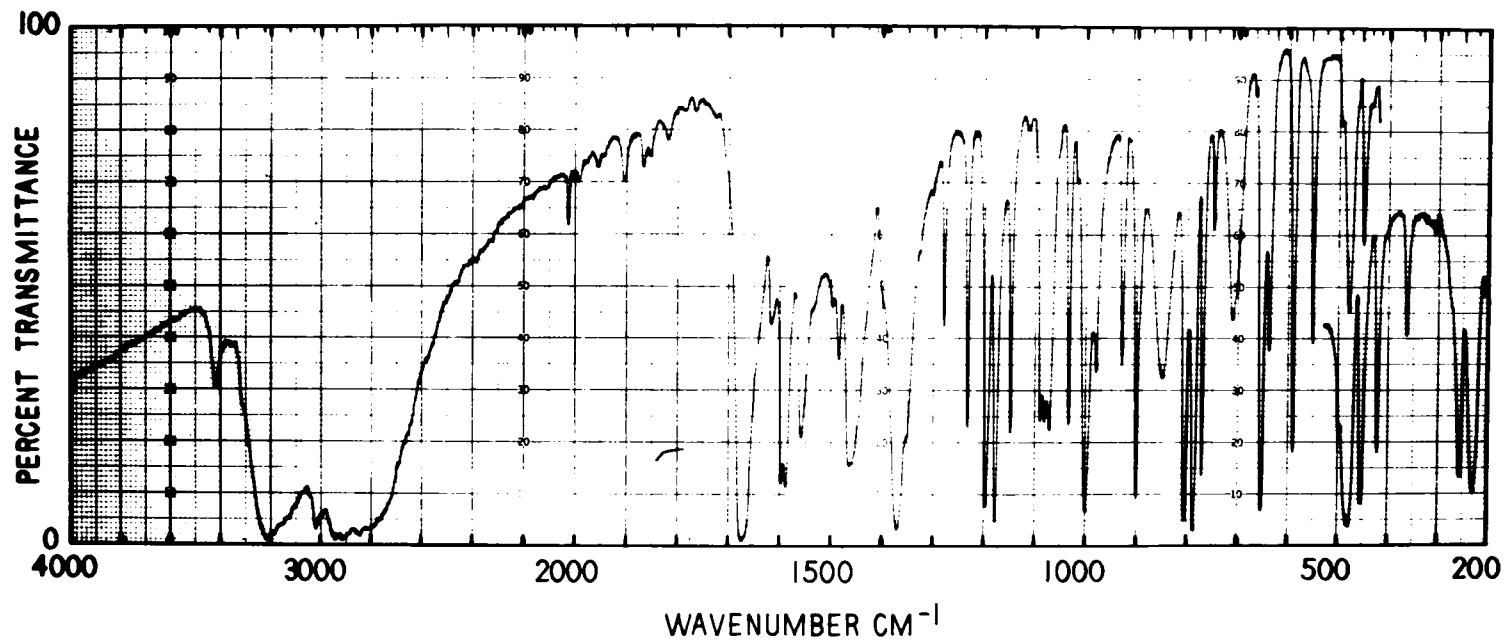


Figure 1. Infrared Absorption Spectrum of Hydralazine Hydrochloride, Mineral Oil Mull.

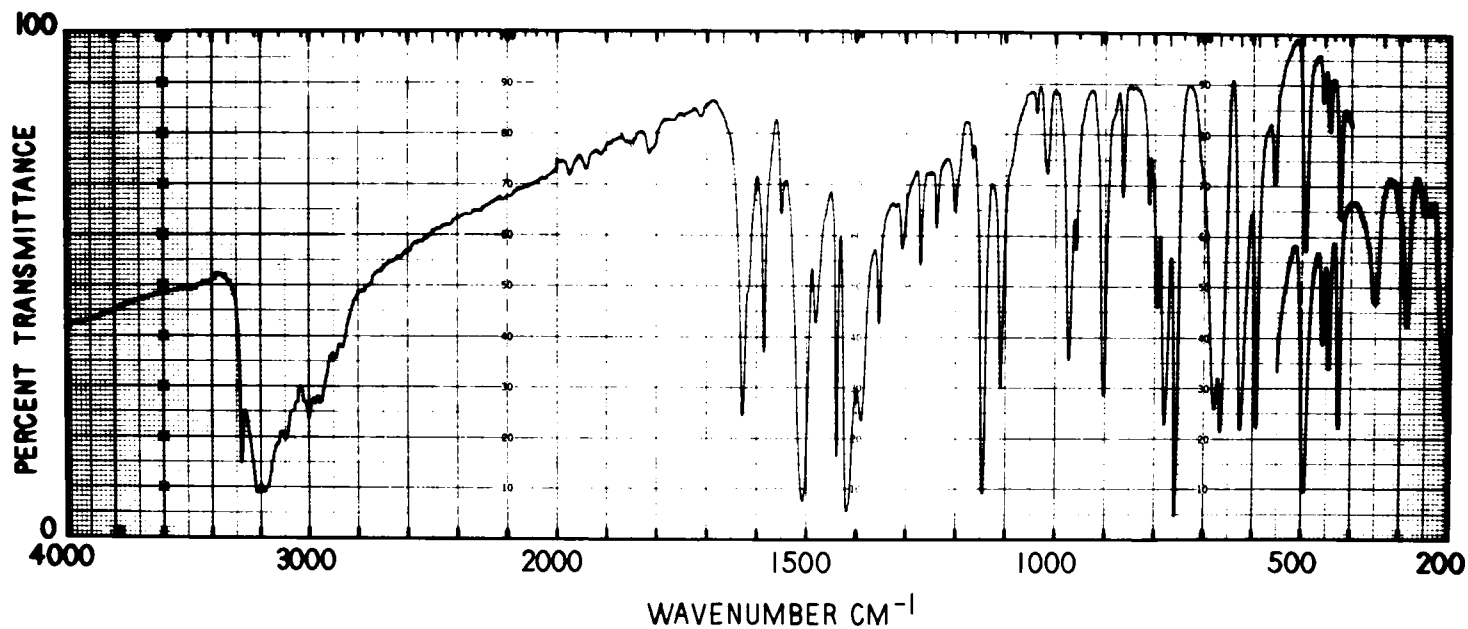


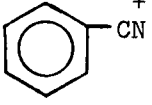
Figure 2. Infrared Absorption Spectrum of Hydralazine Base, Potassium Bromide Pellet and Mull.

219 nm, and 1.0 to 2.0 for the wavelength range 219 to 200 nm. A scan of water against water is also recorded on the chart. The spectrum exhibits maxima at 315, 303, 260, 239, and 211 nm, with respective absorptivities as follows: 4,200; 5,200; 10,600; 11,000; 33,800 l./mole cm.

Druey and Tripod (3), Kuhnert-Brandstätter et al (7), Sharkey et al (8), and Solomonova et al (9) reported similar maxima and absorptivities, except that they did not report the 211 nm maximum. Clarke (6) gives data similar to that of the above investigators. Sunshine (10) gives the spectrum in 0.1N hydrochloric acid, with maxima at 312, 302, 259, and 233 nm, and the spectrum in 0.1N sodium hydroxide, with maxima at 304, 271, and 262 nm. Naik et al (11) gave partial ultraviolet spectra of the base, the monohydrochloride, and the dihydrochloride: (a) the base exhibited maxima at 305 and 273 nm, with a third maximum near 262 nm; (b) the monohydrochloride exhibited maxima at 315, 302, 290, and 260 nm; (c) the dihydrochloride exhibited a maximum at 318 nm with a broad shoulder at about 300 nm. The Merck Index (12) lists maxima at 315, 304, 260, 240, and 211 nm for the aqueous solution of the hydrochloride.

2.3 Mass Spectrum

Figure 4 shows the low resolution mass spectrum of hydralazine hydrochloride. The data were obtained with an LKB 9000S mass spectrometer, with an ionization voltage of 70 eV, source temperature 250°C. Some of the peaks may be assigned as follows (13):

<u>Mass Charge Ratio</u>	<u>Assignment</u>
160	M^+
131	$M^+ - \cdot N=NH$
129	$M^+ - \cdot NH-NH_2$
103	
76	$C_6H_4^+$
51	$C_4H_3^+$
36/38	HCl^+

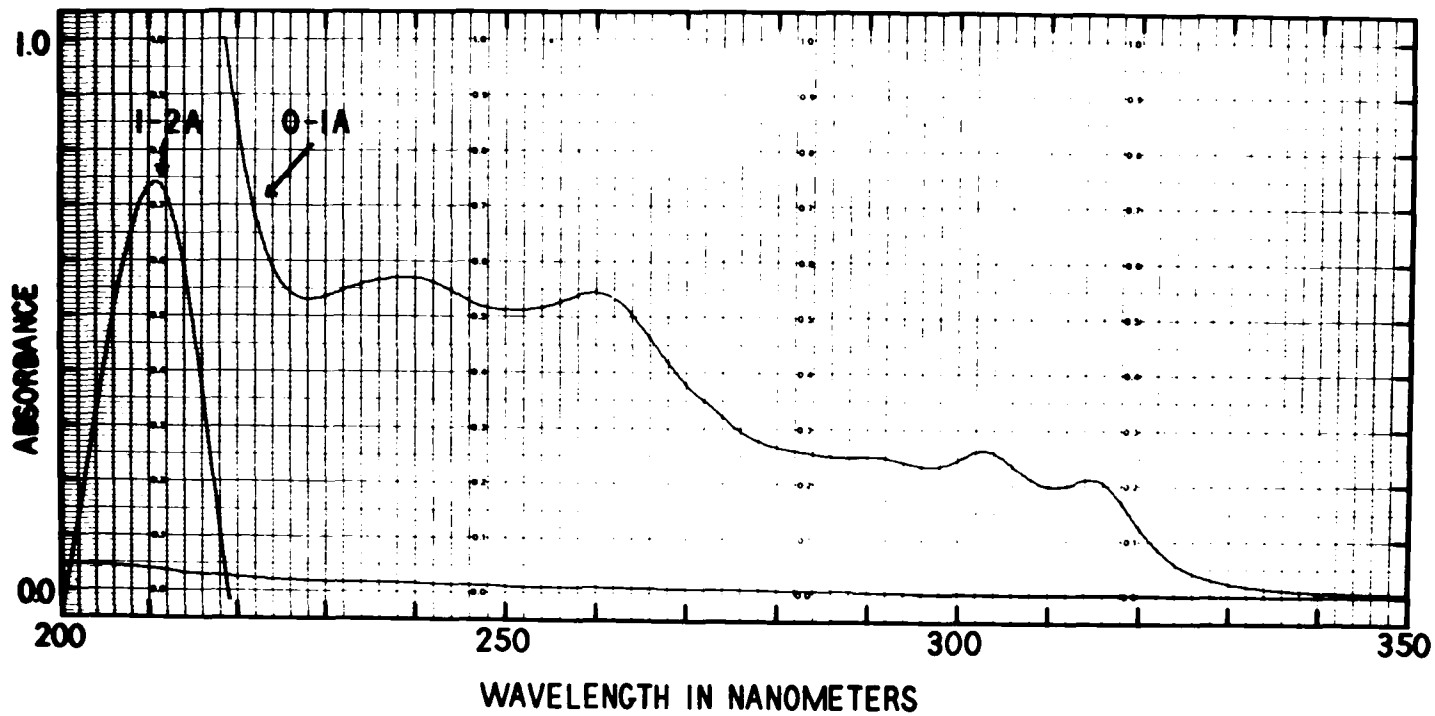


Figure 3. Ultraviolet Absorption Spectrum of Hydralazine Hydrochloride, 9.9 mg per liter, Aqueous Solution.

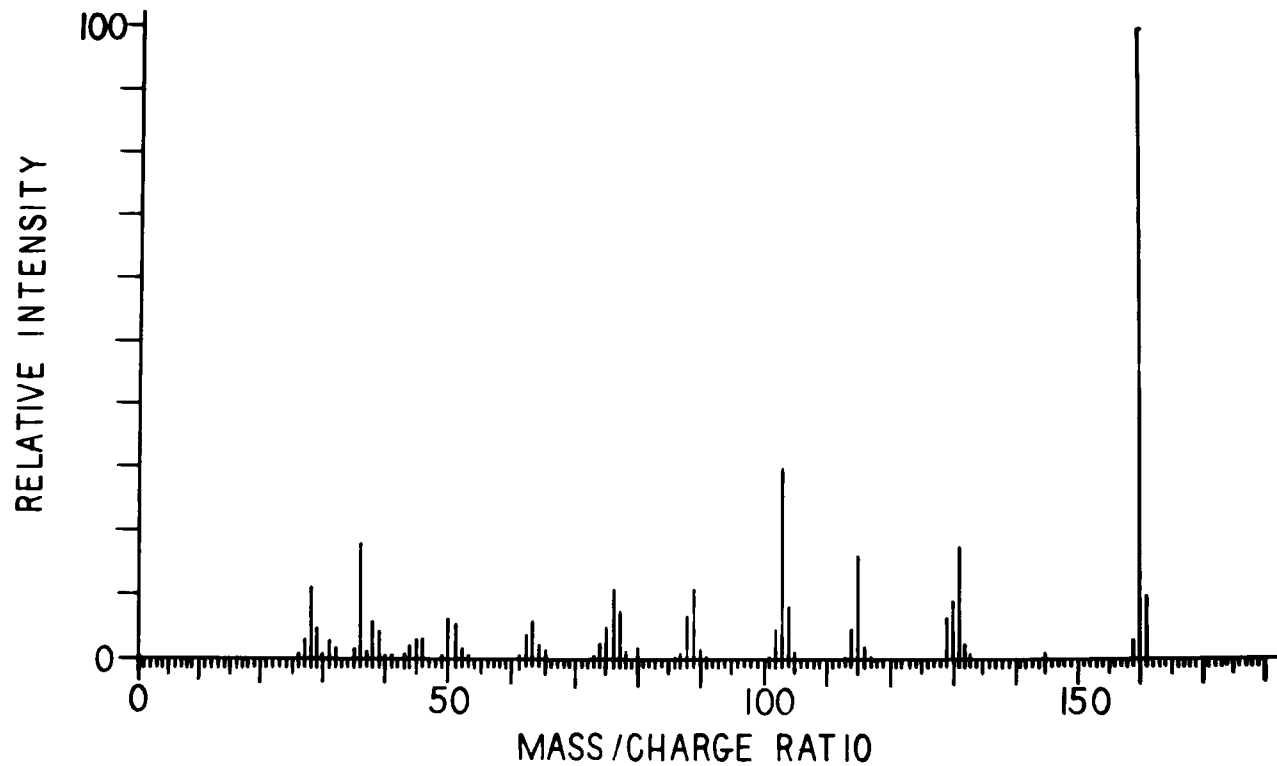


Figure 4. Mass Spectrum of Hydralazine Hydrochloride.

2.4 Nuclear Magnetic Resonance Spectra

The NMR spectrum shown in Figure 5 was obtained by dissolving hydralazine hydrochloride in deuterium oxide containing 3-(trimethylsilyl)-1-propane-sulfonic acid sodium salt (DSS). The series of peaks at 0, 0.6, 1.8, and 3 ppm are all due to the DSS. The peak at 4.8 ppm is due to HDO which forms on exchange with the solvent and the peaks at 8.01 and 8.61 ppm are due to the aromatic protons. The NMR spectrum of the base (Figure 6) was obtained in a 1:1 mixture of dimethylsulfoxide- d_6 :deuteriochloroform. The peaks at 2.53 ppm are due to the solvent. The N-H protons are at 4.1 ppm and the aromatic protons are at 7.6 and 8 ppm. The spectra were produced using a Varian EM-360 NMR spectrometer.

2.5 Differential Thermal Analysis (DTA)

The DTA curves in Figures 7 and 8 were obtained with a DuPont Model 900 instrument. Figure 8 shows that the base melts sharply and Figure 7 shows that the hydrochloride melts with decomposition. Visually the hydrochloride melts at 274°C, but decomposition takes place over a large temperature range (Figure 7).

2.6 Crystal Properties

Chojnacki et al (14) investigated the crystal properties of hydralazine hydrochloride. Recrystallized from water, the crystals were found to be monoclinic, with the unit cell $a = 9.408$, $b = 14.529$, $c = 6.643\text{\AA}$, $\beta = 103.59^\circ$, $Z = 4$. The calculated density was 1.486, the measured density 1.479 g per cm^3 . The space group was $P2_1/c$. Powder diffraction data, generally similar to that of Table 1, is also given.

X-ray powder diffraction data for hydralazine hydrochloride and hydralazine base are given in Table 1. These data were obtained with a Norelco diffractometer using nickel-filtered copper $K\alpha$ radiation.

2.7 Microchemical Characterization

Sandri (15) reported the formation of characteristic crystals with cadmium bromide-hydrobromic acid, bismuth iodide-potassium iodide, and bismuth bromide-hydrobromic acid, with compositions $\text{CdBr}_2 \cdot \text{HBr} \cdot \text{C}_8\text{H}_8\text{N}_4 \cdot 4\text{H}_2\text{O}$, $\text{HBI} \cdot \text{C}_8\text{H}_8\text{N}_4$, and $\text{HBI} \cdot \text{C}_8\text{H}_8\text{N}_4$, respectively.

Kuhnert-Brandstätter et al (7) reported the microscopic hot-stage characterization of hydralazine hydrochloride. Above 210°C, small rods, granules, and prisms sublime. The melting point is 268-275°C. Gas bubbles are evolved as melting begins, and melting proceeds with

TABLE 1
X-RAY POWDER DIFFRACTION PATTERNS OF
HYDRALAZINE HYDROCHLORIDE AND HYDRALAZINE BASE

<u>Hydrochloride</u>		<u>Base</u>	
$d(\text{\AA})$	I/I_0	$d(\text{\AA})$	I/I_0
7.74	100	7.37	100
7.25	93	5.87	11
5.92	9	5.62	49
5.54	2	5.04	11
4.82	2	4.86	17
4.62	18	4.48	18
4.37	47	3.98	2
3.99	15	3.74	10
3.87	12	3.66	2
3.63	54	3.47	15
3.39	9	3.28	100
3.38	11	3.24	17
3.32	31	3.12	11
3.29	65	2.96	2
3.22	92	2.80	7
3.15	7	2.71	1
3.05	9	2.67	2
2.99	4	2.60	2
2.95	2	2.49	2
2.93	2	2.44	2
2.85	6	2.38	1
2.77	22	2.30	2
2.72	2	2.28	1
2.64	2	2.25	2
2.58	4	2.18	1
2.44	7	2.12	3
2.41	2	2.07	2
2.39	1	2.04	3
2.34	16	1.99	1
2.24	2	1.95	2
2.22	2	1.87	2
2.18	2	1.85	1
2.16	2	1.83	1
2.10	3	1.79	1
2.06	2	1.78	1
2.02	1	1.76	1
1.99	2	1.72	3
1.97	4	1.67	1
1.94	4	1.62	1
1.89	2		

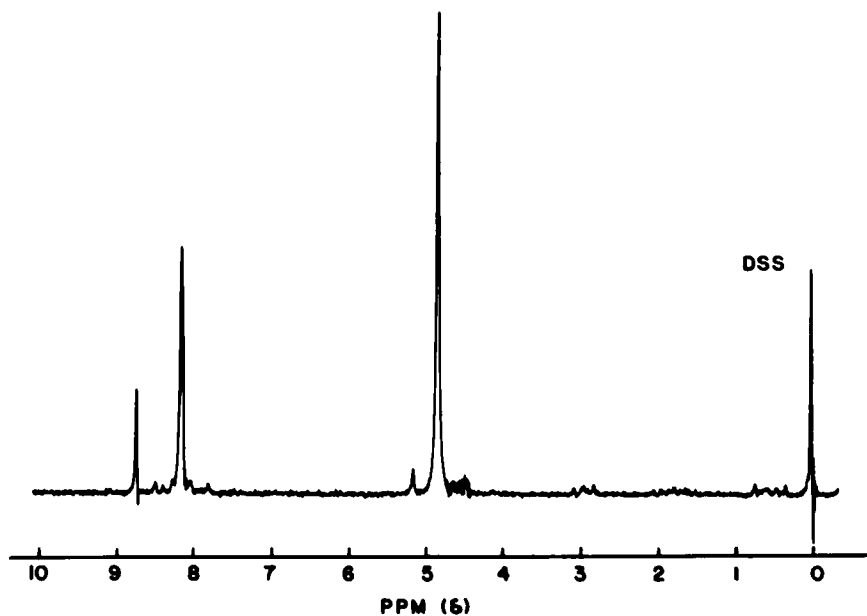


Figure 5. Nuclear Magnetic Resonance Spectrum of Hydralazine Hydrochloride.

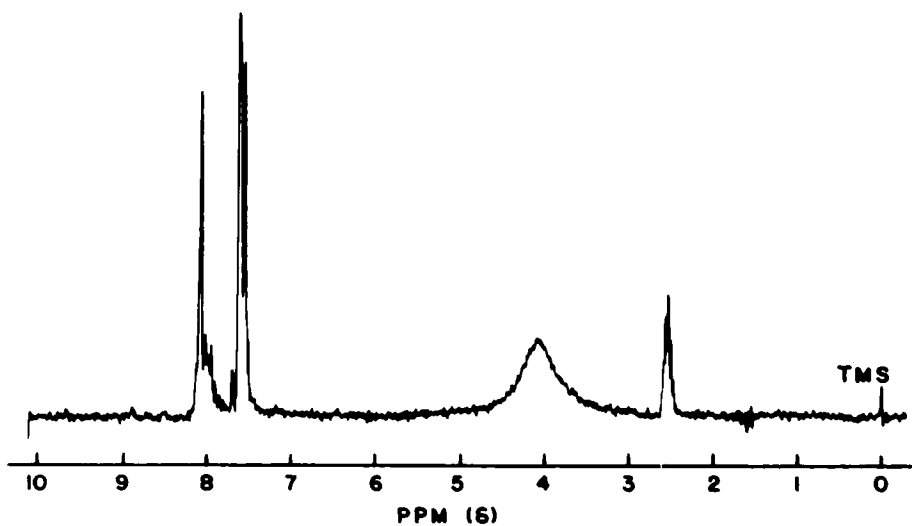


Figure 6. Nuclear Magnetic Resonance Spectrum of Hydralazine Base.

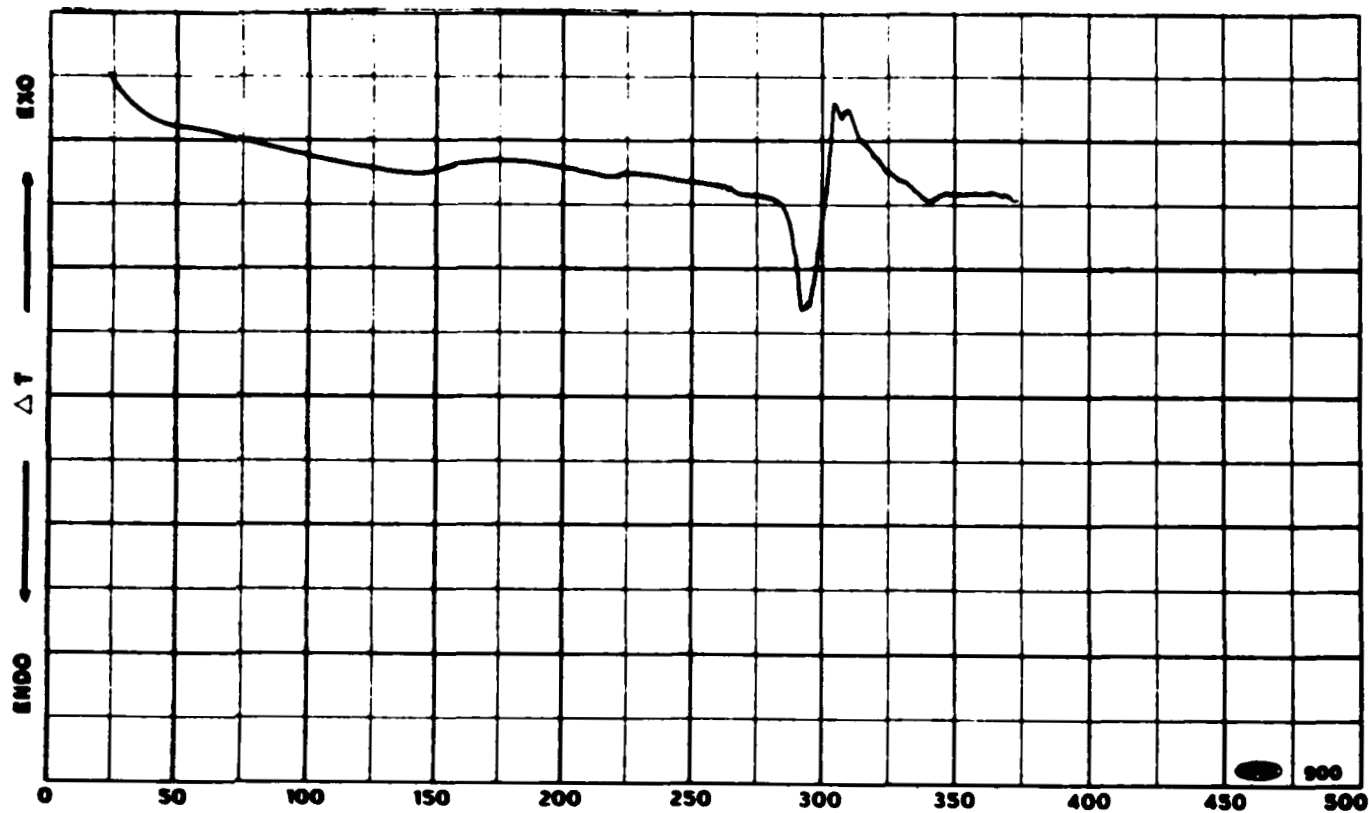


Figure 7. Differential Thermal Analysis Curve of Hydralazine Hydrochloride.

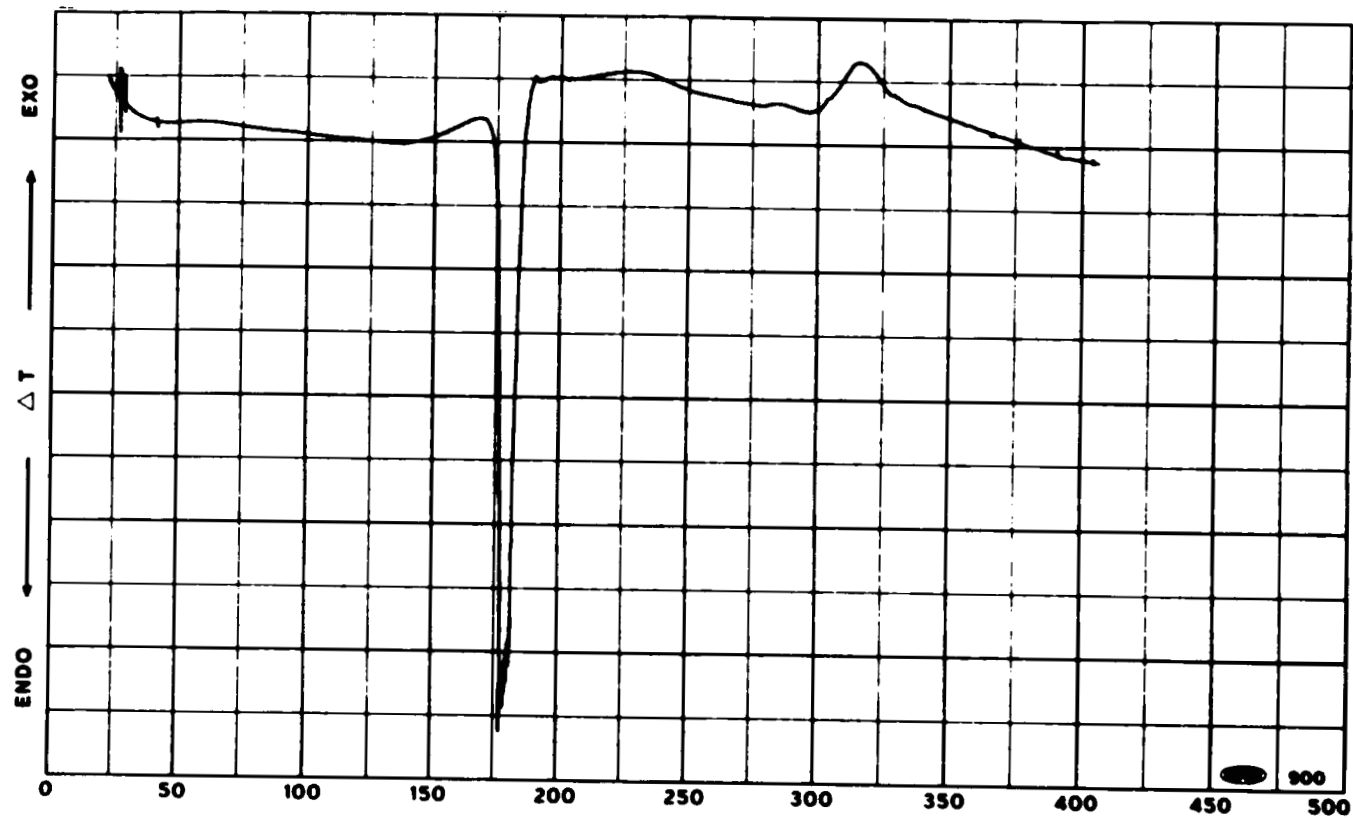


Figure 8. Differential Thermal Analysis Curve of Hydralazine Base.

the separation of high melting (over 300°C) needles and bushlike aggregates. The eutectic temperature with p-acetamidophenyl salicylate was 181°C, with dicyandiamide 162°C. The picrate salt forms from aqueous solution as long needles and columns that melt at 210 to 212°C with decomposition. The styphnate forms needles and rosettes with a melting point of 200 to 203°C.

Sunshine (10) and Clarke (6) give two micro-crystal tests, both from aqueous solution: (a) lead iodide-potassium acetate reagent forms dense rosettes; (b) iodine-potassium iodide (1:50) reagent forms clusters or masses of needles.

2.8 Solubility

The Merck Index (12) gives the solubility in water as 30.1 mg per ml at 15°C, and 44.2 mg per ml at 25°C. In 95% ethanol, the solubility is given as 2 mg per ml. These are consistent with the following approximate solubility data, determined at room temperature:

<u>Solvent</u>	<u>Approximate Solubility, mg/ml</u>
Water	39.
Methanol	6.7
Ethanol (95%)	1.9
2-Propanol	0.1
Chloroform	< 0.1
Ethyl ether (anhydrous)	< 0.1
Ethyl acetate	< 0.1
Acetonitrile	< 0.1

2.9 Dissociation Constants

Evstratova and Ivanova (16) reported a pK_a of 7.1. Evstratova et al (17) reported a pK_a of 7.1 in water, a pK_a of 4.7 in aqueous 90 percent acetone, and a pK_b of 15.6 in the latter solvent. Naik et al (11) reported a pK_a of 6.9 for the dissociation of the monohydrochloride and a pK_a of 0.5 for dissociation of the dihydrochloride, determined absorptiometrically. Artamanov et al reported a pK_a of 7.2 (18).

2.10 Melting Points

The melting point of hydralazine hydrochloride is near 273°C, that of the base is near 173°C. Melting points reported in the literature are as follows:

<u>Melting Point of Hydrochloride (a), °C</u>	<u>Reference</u>
270 - 280	(3,19)
273	(2,20,21,22,23)
271 - 272	(24)
265	(25)
273 - 274	(26)

(a) The hydrochloride melts with decomposition.

<u>Melting Point of Base, °C</u>	<u>Reference</u>
172 - 173	(2,21,22)
165 - 172	(24)
172	(25)
173 - 175	(27)

3. SYNTHESIS

Hydralazine has been prepared by various procedures from 1-chlorophthalazine (2,21,22,24,28), phthalazine-1-thione (20,23,25), 1,4-dichlorophthalazine (29,30,31), 1-(methylsulfonyl)-phthalazine (27), or 1-cyano-2-benzal acetate (26). Phthalazone, used to prepare 1-chloro-phthalazine, has been prepared from naphthalene (3,28) or from phthalic anhydride (3). A comprehensive survey on hydrazinophthalazines and related compounds has been published by Druey and Tripod (3). Some of these procedures are outlined in Figures 9 and 10.

4. STABILITY - DEGRADATION

Hydralazine hydrochloride is quite stable as the crystalline solid. At room temperature, it is stable in distilled water solution for weeks (32). In aqueous

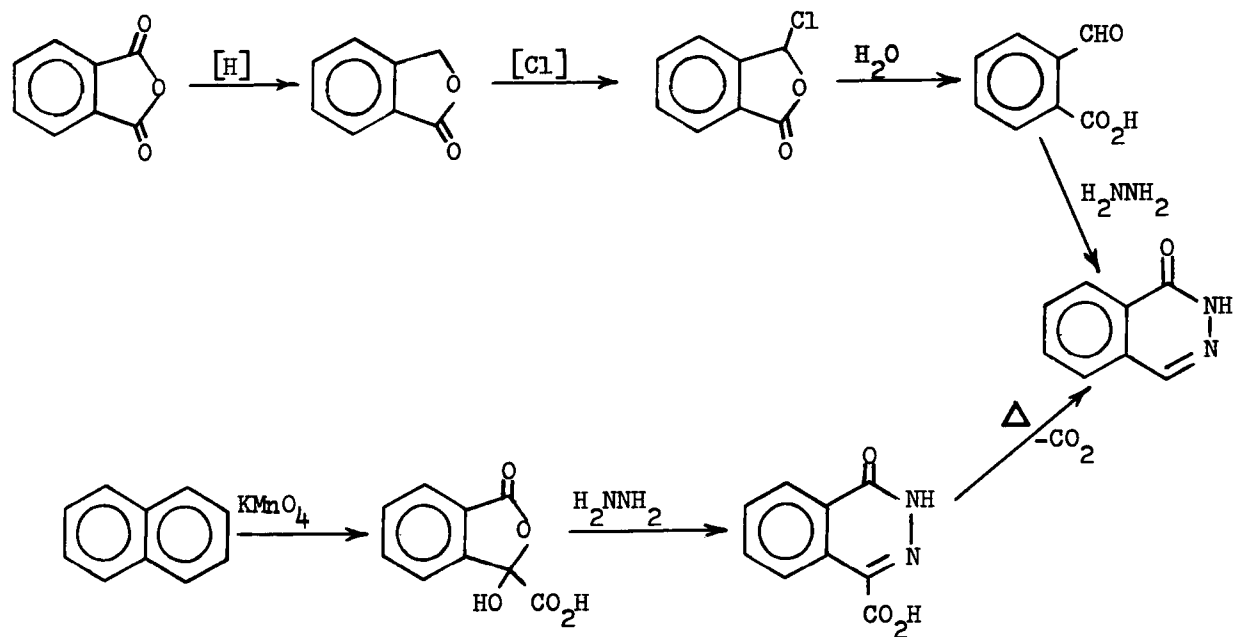


Figure 9. Syntheses of Phthalazone.

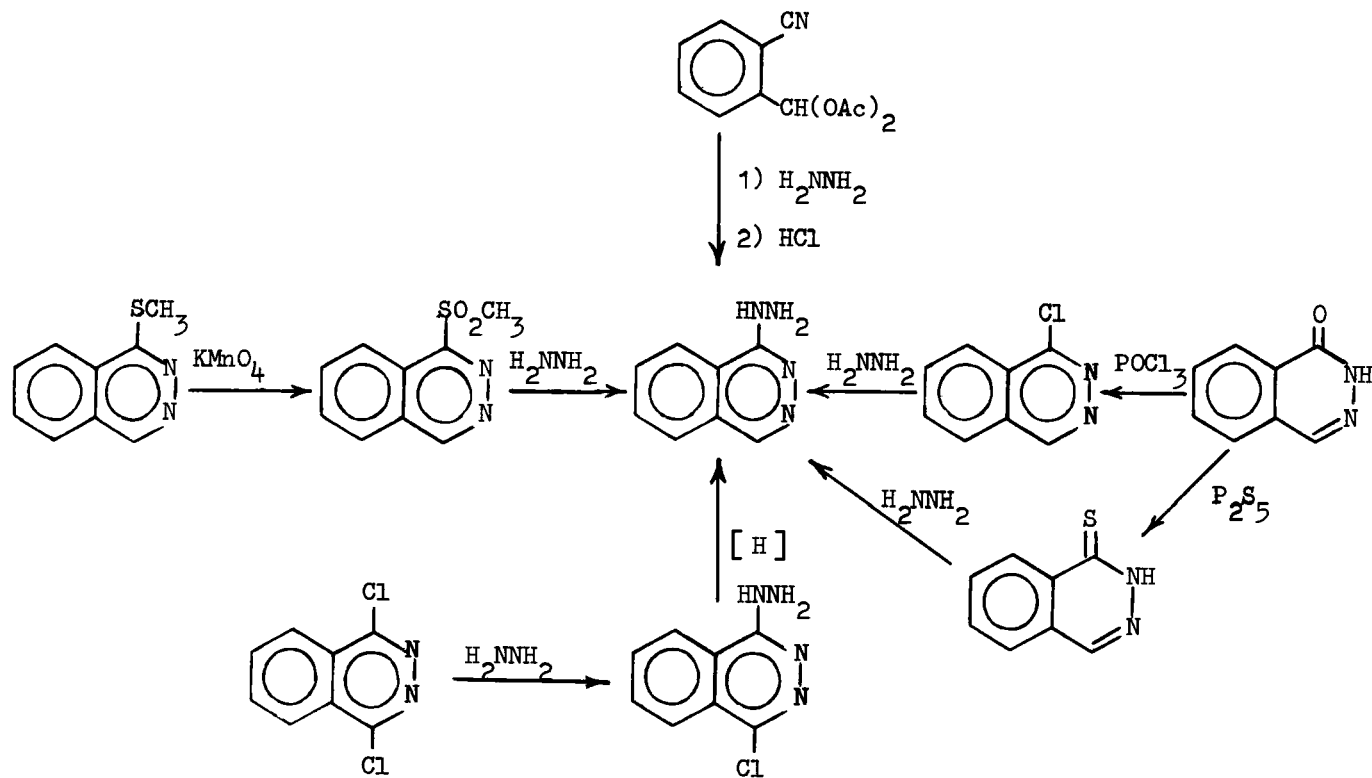


Figure 10. Several Syntheses for Hydralazine.

solution with a pH greater than 7, hydralazine decomposes, forming phthalazine; the rate of decomposition depends on pH, temperature, and the kind and concentration of anion present (33).

In biological samples the drug disappears rapidly at room temperature, apparently by enzymatic reactions.

The hydrazino group is highly reactive, forming hydrazones with aldehydes and ketones. It is also a reducing agent, and it forms complexes with many metal ions (34).

At 275 to 280°C it decomposes to hydrazine, ammonia, nitrogen, and 1,4-dihydro-1,1'-biphtalazine (35).

5. METABOLISM

Hydralazine hydrochloride is rapidly metabolized and excreted. Experiments with carbon-14 labeled drug in humans indicated that less than 10 percent of the intact drug was excreted (36). Within 5 days after a dose, 83 to 89 percent was excreted in the urine and 9 to 12 percent in the feces. Of the material excreted in the urine, 96 percent was recovered in the first 24 hours. Individuals who are slow acetylators exhibit higher hydralazine blood levels than fast acetylators, for the same dose (37).

Reported metabolic products of hydralazine in various species are as follows:

	<u>Metabolite</u>	<u>Species</u>	<u>Reference</u>
I.	3-Methyl-s-triazolo [3,4a]phthalazine	Man	(36,37,38,39, 40,41,42,43,44)
		Rat	(38,39,43,45, 46)
		Guinea pig	(45)
		Rabbit	(38)
		Pigeon	(45)
II.	N-(1-Phthalazinyl)- hydrazone of pyruvic acid	Man	(36,38,47,85)
		Rat	(38,46)
		Rabbit	(38)
III.	N-(1-Phthalazinyl)- hydrazone of acetone	Man	(47)
		Rat	(44,46)
IV.	N-(1-Phthalazinyl)- hydrazone of α - ketoglutaric acid	Man	(47)

V.	s-Triazolo[3,4a] phthalazine	Man Rat	(36,43,47,48) (46)
VI.	1(2H)-Phthalazinone	Man Rat	(36,43,48) (46)
VII.	Phthalazine	Man Rat	(37) (46)
VIII.	4-(2-Acetylhydrazino)- phthalazinone	Man	(36)
IX.	9-Hydroxy-3-methyl-s- triazolo[3,4a] phthalazine	Man	(36)
X.	3-Hydroxymethyl-s- triazolo[3,4a] phthalazine	Man Rat	(36,43) (46)

In cases where the metabolites above possess suitable functional groups, they are usually excreted as glucuronides or sulfates.

Figure 11 is a scheme illustrating the metabolism of hydralazine, based primarily on those proposed by Haeghele et al (46) and Wagner et al (36).

6. METHODS OF ANALYSIS

6.1 Identification Tests

Hydralazine hydrochloride can be easily identified by the physical properties described in Section 2 above.

Where identification of hydralazine in formulations is necessary, the following tests may be useful.

Cooper (49) used spot tests on paper, reacting ferric ion or dimethylaminobenzaldehyde with the unknown. The iron reagent yields a blue color, and dimethylaminobenzaldehyde an orange color. The results obtained with 95 other drugs are given.

Belikov et al (50) described three reactions to identify hydralazine. Sodium nitroprusside reacts with an alkaline aqueous solution of hydralazine to yield a red color; when the mixture contains mineral acids, a green precipitate is formed, but with acetic acid, a red precipitate is formed. Cinnamaldehyde reacts with a hydrochloric acid solution of hydralazine to give a yellow precipitate, m.p. 197-200°C. p-Nitrobenzaldehyde reacts with an

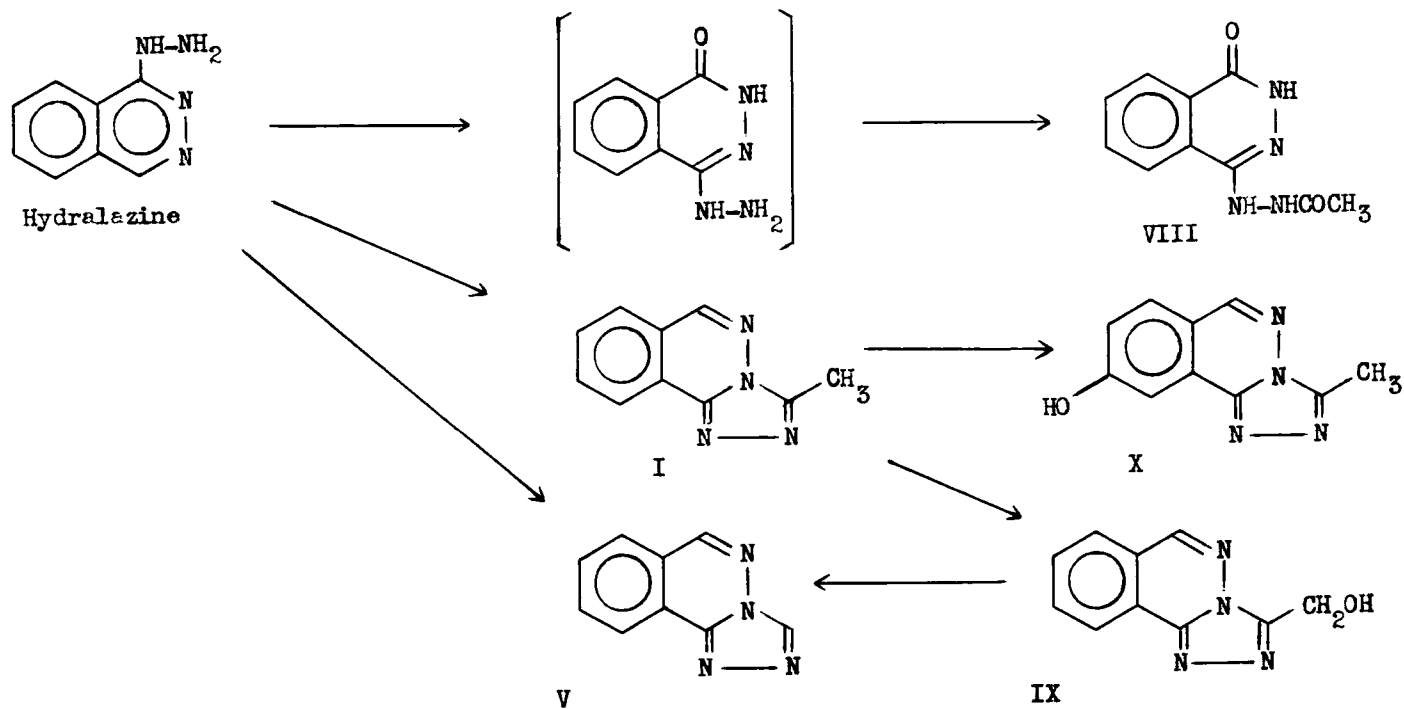


Figure 11a. Metabolic Products of Hydralazine.

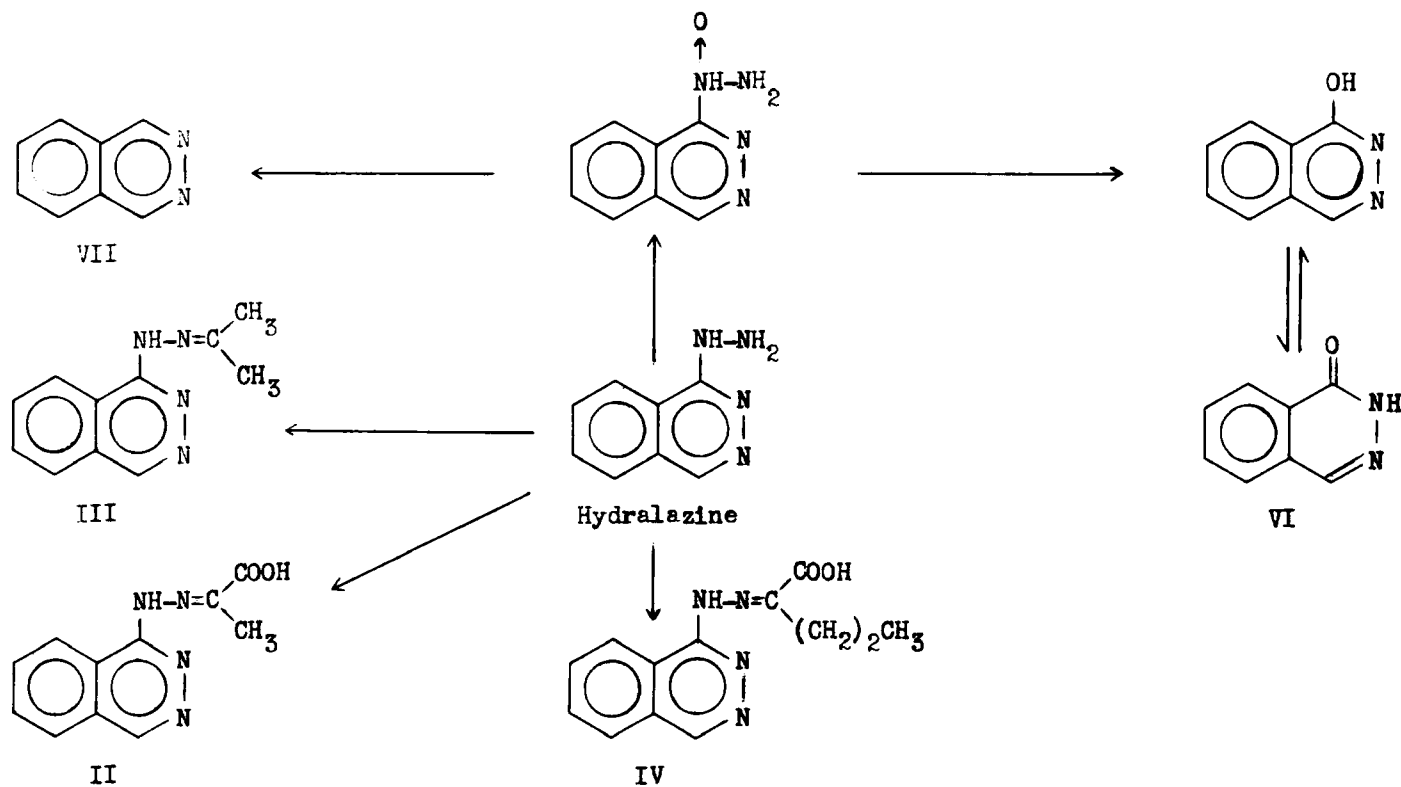


Figure 11b. Metabolic Products of Hydralazine.

aqueous solution of hydralazine to give an orange precipitate, m.p. 260-261°C.

Modras (51) reported spot test reactions to differentiate hydralazine from closely related drugs. Reagents used were aqueous copper (I) chloride, aqueous ammonium molybdate, iodine in potassium iodide solution, aqueous cobalt (II) nitrate, alcoholic ninhydrin, and alcoholic bromophenol blue. The tests were performed on paper or on Silica Gel G.

Roshchenko et al (52) reacted hydralazine with p,p'-dichlorobenzene sulfonamide sodium in water at 80 to 90°C to produce a crystalline precipitate $C_{20}H_{17}O_4N_5S_2Cl_2$ with a melting point of 196-197°C.

In the USP (19) method for identifying hydralazine in tablets and injections, o-nitrobenzaldehyde is added to form an orange precipitate.

Stohs and Scratchley (53) used thin layer chromatography to identify hydralazine in dosage forms and biological fluids (see Section 6.8).

6.2 Elemental Analyses

The elemental compositions of hydralazine hydrochloride and hydralazine base are as follows:

<u>Element</u>	<u>Hydrochloride</u>		<u>Base</u>	
	<u>Calculated</u>	<u>Found(2)</u>	<u>Calculated</u>	<u>Found(2)</u>
Carbon	48.87	48.99	59.99	- - -
Hydrogen	4.61	4.83	5.03	- - -
Nitrogen	28.49	28.39	34.98	35.09
Chlorine	18.03	18.17	- - -	- - -

6.3 Spectrophotometric Methods

Solomonova et al (9) determined hydralazine in tablets by a direct ultraviolet absorption method.

Although the compound can be extracted from formulations with alcohol or water, other components of the mixture may interfere with direct ultraviolet absorption measurement by contributing to the observed absorption. The solutions usually cannot be cleaned up by extraction techniques because hydralazine decomposes in alkaline solution. However, there are many reactions that give rise to near ultraviolet or visible absorption bands suitable for quantitation.

A frequently reported spectrophotometric technique for the determination of hydralazine is based on reactions with aromatic aldehydes to form hydrazones with absorption in the visible region. Luk'yanchikova et al (54) used p-nitrobenzaldehyde; Wesley-Hadzija and Abaffy (55) and Ruggieri (56) used p-dimethylaminobenzaldehyde; Luk'yanchikova (57,58) used cinnamaldehyde; Schulert (33) used p-hydroxybenzaldehyde; and Zak et al (59) used p-methoxybenzaldehyde, after testing cinnamaldehyde, salicylaldehyde, 3,4,5-trimethoxybenzaldehyde, and 1-naphthaldehyde.

Perry (32), and Grabowicz and Brulinska (60) used ninhydrin to obtain a solution with an absorption maximum near 450 nm.

Ellert and Modras (61) treated hydralazine with ferrous ion in alkaline solution, and measured the color produced at 540 nm.

Ruggieri (56) reported a colorimetric test using 2-naphthoquinonesulfonate sodium to form a rose-violet color.

Urbanyi and O'Connell (62,63) developed an automated colorimetric method using blue tetrazolium for the analysis of hydralazine in the presence of reserpine and hydrochlorothiazide.

6.4 Fluorescence

Naik et al (11) extracted hydralazine hydrochloride from tablets with 50 percent aqueous methanol and mixed a portion of the extract with 99 volumes of concentrated sulfuric acid to obtain fluorescence at 353 nm with excitation at 320 nm. The fluorescence intensity varied linearly with concentration in the range 2 to 8 μg hydralazine hydrochloride per ml. Injections were analyzed similarly.

6.5 Titration Methods

Ruggieri (56) reported titrating hydralazine with perchloric acid.

Ruzhentseva et al (64) converted hydralazine to ammonia by heating with zinc in sulfuric acid, forming 3 moles of ammonia per mole of hydralazine. The mixture was made alkaline and the ammonia was distilled into boric acid solution, which was then titrated.

Sandri (15) tested three titration methods with good results. One was titration with potassium bromate in the presence of potassium bromide and hydrochloric acid, using a starch-iodine end-point. Another was addition of excess periodic acid-potassium iodide with sodium

thiosulfate back-titration. The third was addition of sodium hydroxide and a concentrated solution of potassium ferrocyanide, then acidifying and titrating with potassium permanganate solution.

Perel'man and Evstratova (65) titrated hydralazine hydrochloride in dimethylformamide solution to a potentiometric end-point with sodium methoxide solution.

Artamanov et al (18) determined hydralazine hydrochloride by conductometric titration with sodium hydroxide solution.

Goryacheva and Prikhodkina (66) titrated hydralazine from tablets with N-bromosuccinimide solution. One mole of hydralazine hydrochloride was equivalent to 2 moles of N-bromosuccinimide. Methyl red was used as the indicator.

Soliman and Belal investigated argentimetric (67,68) and mercurimetric (69) methods. Hydralazine precipitates silver from ammoniacal silver nitrate solution. The silver is dissolved with hot nitric acid and titrated with ammonium thiocyanate solution. Alternatively, mercury is precipitated from alkaline potassium mercuric iodide solution. The precipitated mercury is dissolved by adding excess standard iodine solution. The excess iodine is back-titrated with sodium thiosulfate solution after acidifying with acetic acid.

USP XIX directs that hydralazine be determined in the raw material, tablets, and injections by potassium iodate titration in strongly acid solution, using chloroform to detect the presence of iodine (19).

6.6 Gasometric Methods

McKennis and Yard (70) studied the nitrogen evolution from a series of hydrazino compounds when treated with $0.02M$ KIO_3 in $0.2N$ H_2SO_4 in a Warburg apparatus at $37^\circ C$. In an aqueous solution, hydralazine released 102 percent of the theoretical amount of nitrogen in 15 minutes. Viala (71) determined hydralazine in solutions or tablets by measuring the nitrogen freed from the hydrazine group, using potassium permanganate in sulfuric acid, or iodine in sodium bicarbonate solution.

6.7 Polarography

Polarographic studies of hydralazine and related compounds were reported by Giovanoli-Jakubczak et al (72) and by Modras (73). The reduction proceeds in two 2-electron stages with the formation of the tetrahydro derivative. Hydralazine could be determined in the

presence of decomposition products, at a concentration of about 0.001M in 1N hydrochloric acid containing a small amount of gelatin. The half-wave potentials of the two waves were -0.7 and -0.95 V against a saturated calomel electrode.

6.8 Paper Chromatography

Ruggieri (56) and McIsaac and Kanda (38) describe chromatography on Whatman No. 1 paper with butanol/acetic acid/water (4:1:5) as the solvent. Detection was by quenching of the background fluorescence of the paper under ultraviolet light or by ammoniacal silver nitrate. The R_f value for hydralazine was 0.90. Several investigators (41,45,74) have used alkaline systems for paper chromatography of hydralazine and its metabolites, but Lesser et al (75) suggest that alkaline chromatography systems may not be suitable for hydralazine itself.

6.9 Thin Layer Chromatography

Stohs and Scratchley (53) investigated several thin layer chromatography systems for thiazide diuretics and antihypertensive drugs, using silica gel G and a variety of detection reagents.

<u>Solvent System</u>	<u>Hydralazine R_f</u>
Methyl ethyl ketone/n-hexane (1:1)	0.72
Methyl ethyl ketone/n-hexane (2:1)	0.62
Methyl ethyl ketone/n-hexane (3:1)	0.00
Chloroform/acetone/triethanolamine (50:50:1.5)	0.68

Reagents for detecting hydralazine were Dragendorff's reagent, alkaline dimethylaminobenzaldehyde, anisaldehyde, and Bratton-Marshall reagent.

Zak et al (59) used 3N hydrochloric acid/methanol ascorbic acid (44:6:1) on silica gel and found an R_f value of 0.53 for hydralazine.

Lesser et al (75) used cellulose sheets with fluorescent indicator, acetic acid/0.01M aqueous disodium edetate (3:97), and observed an R_f value of 0.78 for hydralazine. On silica gel with fluorescent indicator, the R_f value was less than 0.05 with (a) chloroform/n-heptane/acetic acid (6:4:1), and (b) cyclohexane/chloroform/acetonitrile (1:2:1).

6.10 High Pressure Liquid Chromatography

Smith et al (76,77) analyzed hydralazine in a drug mixture containing hydralazine hydrochloride, hydrochlorothiazide, and an impurity derived from the latter. The column was 1 m x 2.1 mm (ID) stainless steel, packed with a strong anion exchanger on 30 μ m Zipax®. The mobile phase was pH 9.2 borate buffer containing 0.005M sodium sulfate (5% methanol), at 1.7 ml per minute. Detection was by ultraviolet absorption at 254 nm.

Honigberg et al (78) tested reversed-phase chromatography for separation of a number of drugs, including hydralazine. The columns contained either octadecyltrichlorosilane or diphenyldichlorosilane, bonded to 37 to 50 μ m pellicular silica packing. Of the numerous mobile phases tested, the best for separating hydralazine, hydrochlorothiazide, and reserpine was acetonitrile/0.1% ammonium acetate (20:80), pH 7.35. The columns were 1.22 m x 2.3 mm (ID) and the flow rate was 1.4 ml per minute. Detection was by ultraviolet absorption at 254 nm.

6.11 Gas Chromatography

Jack et al (79) determined hydralazine in plasma. The sample was treated with nitrous acid, which reacts with hydralazine to form tetrazolo[1,5a]phthalazine (2). The derivative was extracted with benzene and determined by gas chromatography. 1-Hydrazino-4-methylphthalazine was used as an internal standard.

The same procedure, or modifications of it, was used by Zak et al (80), Talseth (42,82,82,83), and Haegele et al (46) for metabolic studies. Zak et al (80) point out that hydrolysis of conjugates of the drug may cause analytical results on biological samples to be variable, depending on the acid concentration during derivatization, and that selective analysis for unchanged hydralazine and acid-labile metabolites can be carried out by suitable adjustment of the acid concentration.

Smith et al (84) determined hydralazine in tablets. An aqueous extract of the tablets was treated with 2,4-pentanedione, forming 1-(3,5-dimethylpyrazole) phthalazine. The method was applied to stability studies of tablets subjected to elevated temperatures, where tablets could not be analyzed by the USP method.

7. ACKNOWLEDGMENTS

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Table 2

GAS CHROMATOGRAPHY SYSTEMS USED FOR DERIVATIZED HYDRALAZINE DETERMINATIONS

<u>Reference Number</u>	<u>Column</u>	<u>Carrier Gas</u>	<u>Column Temp., °C</u>	<u>Detector</u>
(79)	1.5 m x 2 mm ID, glass, packed with 3% OV-225 on 80-100 mesh Chromosorb W-HP	N ₂ , 30 ml/min.	220 °C	Pulsed elec- tron capture (Ni-63, 10 mCi)
(80)	1.2 m x 2 mm ID, glass, packed with 3% OV-225 on Gas-Chrom Q	He, 35 ml/min.	220 °C	Electron capture
(46)	1 m x 4 mm ID, glass, packed with 3% OV-17 on 80-100 mesh Chromosorb W	He, 40 ml/min.	Programmed, at 4°/min., 170-240 °C	Mass spectrometer
(84)	1.8 m x 4 mm ID, glass, packed with 10% SE-30 on 80-100 mesh Gas- Chrom Q	N ₂ , 55 ml/min.	200 °C	Flame ionization

interpretation, the library staff (Ms. J. McDonough and Ms. G. Smith) for the literature search, and Mrs. S. Willette for typing the profile.

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CALCIUM LEUCOVORIN

Leslie O. Pont, Andrew P. K. Cheung, and Peter Lim

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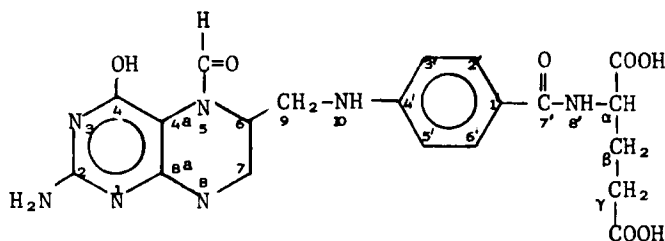
1. Description

1.1 Name, Structure, Empirical Formula, and Molecular Weight

Citrovorum factor (CF), folinic acid, and leucovorin are all N-[4[[[(2-amino-5-formyl-1,4,5,6,7,8-hexahydro-4-oxo-6-pteridinyl)methyl]-amino]benzoyl]glutamic acid, I, the *Leuconostoc citrovorum* 8081* growth factor first reported by Sauberlich and Baumann.¹ Leucovorin refers to the chemically synthesized material that contains both dL and lL diastereomers; "citrovorum factor" and "folinic acid" generally apply to the biologically synthesized lL isomer. Much of the data reported here was obtained on calcium leucovorin, the stable, isolatable, biologically compatible salt.

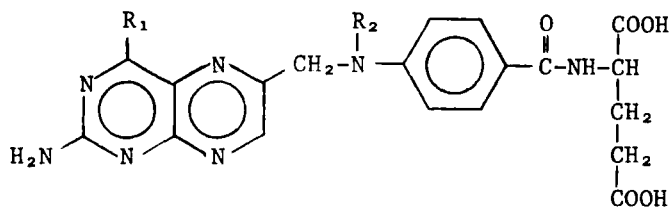
<u>Name</u>	<u>Empirical Formula</u>	<u>Molecular Weight</u>
Leucovorin	$C_{20}H_{23}N_7O_7$	473.449
Calcium leucovorin	$C_{20}H_{21}N_7O_7Ca$	511.513

Calcium leucovorin is also identified by the National Cancer Institute number: NSC 3590.



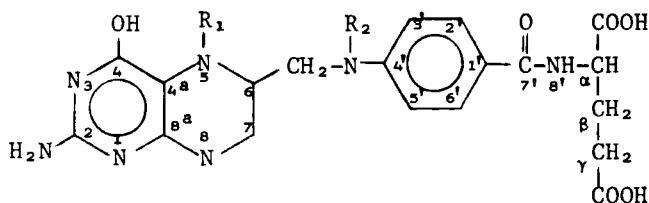
I

Citrovorum factor CF, folinic acid, leucovorin, $5fH_4F$



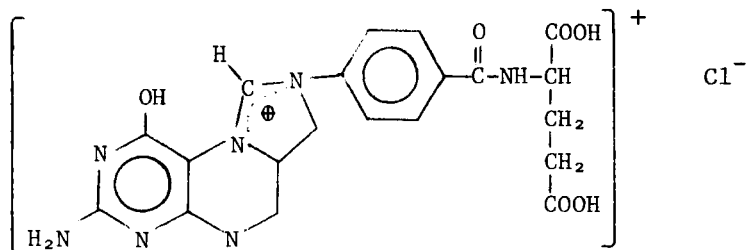
II

- IIa $R_1 = \text{OH}$ $R_2 = \text{H}$ Folic acid, FA
 IIb $R_1 = \text{NH}_2$ $R_2 = \text{CH}_3$ Methotrexate, amethopterin, MTX
 IIc $R_1 = \text{OH}$ $R_2 = \text{CHO}$ 10 formylfolic acid, 10fFA



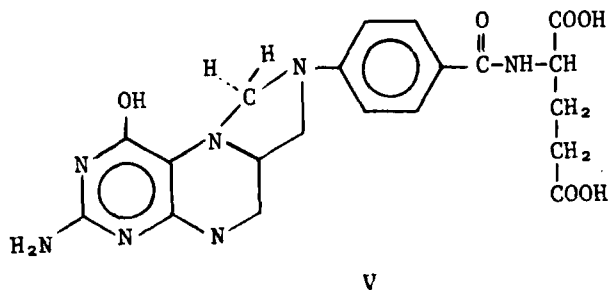
III

- IIIa $R_1 = \text{H}$ $R_2 = \text{H}$ Tetrahydrofolic acid, H_4F
 IIIb $R_1 = \text{CH}_3$ $R_2 = \text{H}$ 5-methyltetrahydrofolic acid, $5\text{mH}_4\text{F}$
 IIIc $R_1 = \text{H}$ $R_2 = \text{CHO}$ 10-formyltetrahydrofolic acid, $10\text{fH}_4\text{F}$



IV

$\text{N}^5, \text{N}^{10}$ -methenyltetrahydrofolic acid, anhydroleucovorin,
 5,10-methenyl H_4F



N^5,N^{10} -methylenetetrahydrofolic acid, 5,10-methylene H_4F

1.2 Appearance, Odor, Color

Calcium leucovorin occurs as an amorphous, odorless powder that is off-white to light beige in color. The salt is not isolated in an anhydrous form, but generally contains 10-15% water (3-5 molecules of hydration).

1.3 Isomeric Forms

Two asymmetric carbon atoms, the α carbon in the glutamic acid portion of the molecule and the C_6 carbon in the tetrahydropteridine ring, allow four possible isomers. Since synthetic procedures would undoubtedly start with L-glutamic acid, the isomeric possibilities are reduced to the dL and lL diastereomers. Of these, the biologically more active form is the lL; separation of the diastereomers is effected by solubility differences of the calcium salts.²

*

Reclassified as *Pedicoccus cerevisiae*.

Although a pure dL sample had not been obtained, a sample possessing a high positive rotation and lower biological activity had been isolated.²

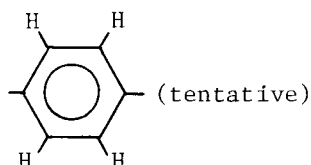
2. Physical Properties

2.1 Infrared Spectrum

Figure 1 is the infrared spectrum taken from a mineral oil suspension of a representative sample of calcium leucovorin. A common feature of folate derivative spectra obtained in this laboratory is the absence of sharp absorption bands, which is usually attributed to lack of crystallinity. Below are listed the major absorption assignments:

<u>Ir absorption (μ)</u>	<u>Assignment</u>
~ 3.0	H ₂ O, N-H(2)
6.0-6.7	H ₂ O, amide I, aryl systems, CO ₂ ⁻ , amide II
~ 7.1	CO ₂ ⁻

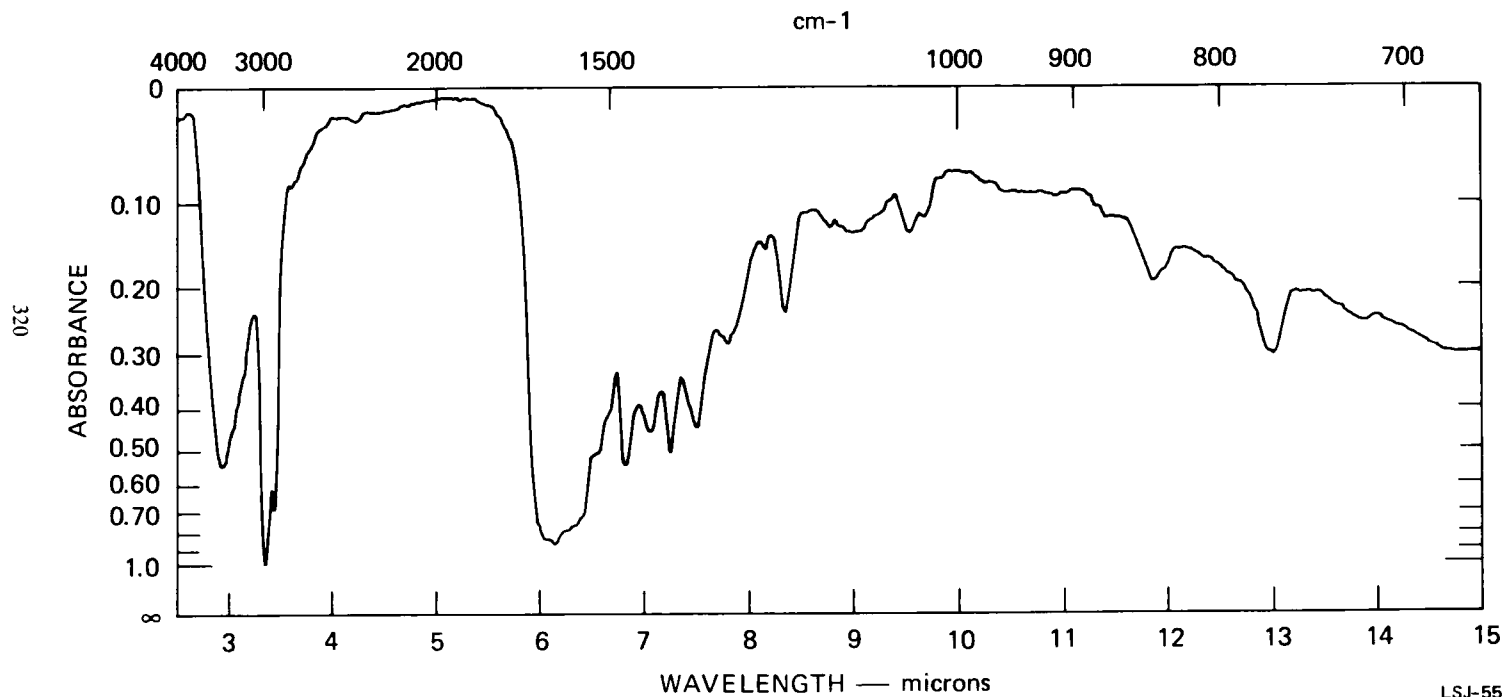
11.9



2.2 Proton Magnetic Resonance Spectrum

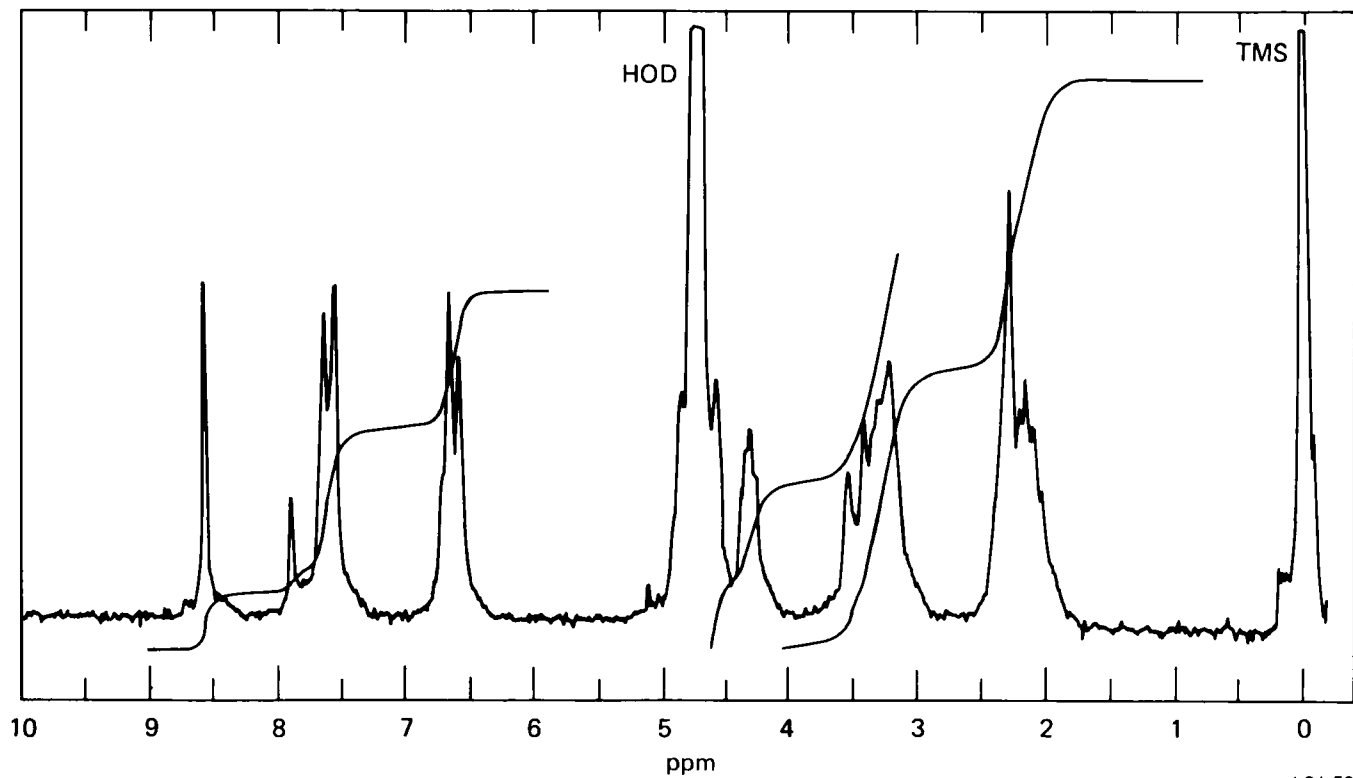
Due to the ionic nature of calcium leucovorin, an ¹H nmr spectrum could not be obtained in an aprotic solvent; therefore, the spectrum was obtained from a deuterated aqueous solution. The following assignments have been made relative to TMS = 0.00 ppm from a 100-MHz Varian XL100 spectrum (Figure 2):

<u>Assignment</u>	<u>Chemical Shift (ppm)</u>	<u>Multiplicity</u>	<u>J (Hz)</u>
H β , γ	1.70-2.60	m	
H 7, 9	2.95-3.80	m	
H α	4.00-4.60 (obscured by HOD)	q	



LSJ-55

FIGURE 1 INFRARED SPECTRUM OF CALCIUM LEUCOVORIN



LSJ-56

FIGURE 2 ^1H NMR OF CALCIUM LEUCOVORIN

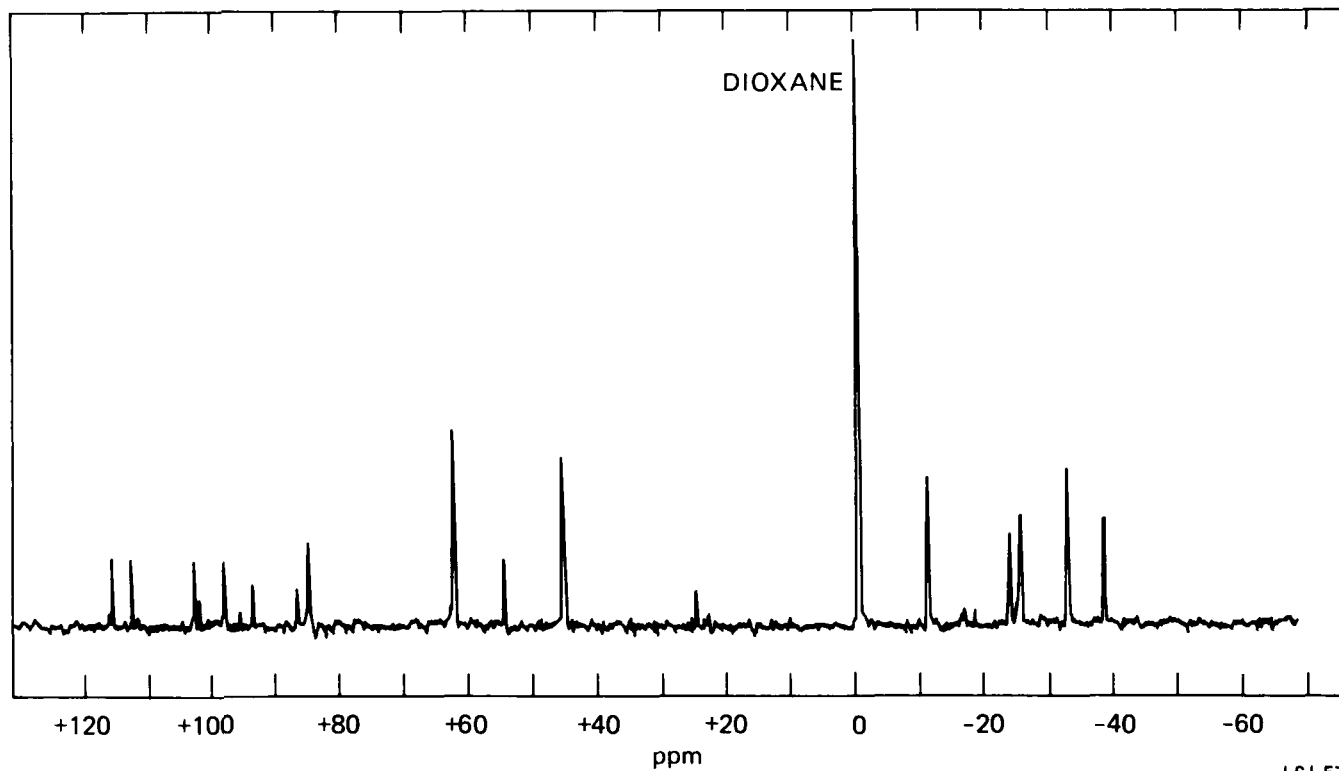
<u>Assignment</u>	<u>Chemical Shift (ppm)</u>	<u>Multiplicity</u>	<u>J (Hz)</u>
H 3', 5'	6.64	d	8
H 2'c 6'	7.60	d	8
$\begin{array}{c} \text{O} \\ \parallel \\ \text{H-C-}, 5 \end{array}$	8.59	s	
unknown	7.89	s	
H, 6	~ 4.8	m (broad)	

The above assignments are in general agreement with those of Pastore³ for folates. The C₆ proton is obscured by the large HOD peak, and its location was confirmed by shifting the HOD absorbance at 80° C. The peak at ~ 7.9 ppm due to an unknown species has been present in all calcium leucovorin samples. The identity of the species responsible for this singlet remains unknown, but at the present time these authors hypothesize that the dL diastereomer's formyl group contributes this peak. Since chemically prepared calcium leucovorin is assayed at this laboratory, an ¹H nmr spectrum has not been obtained on a biologically or enzymatically prepared material. Either should be folinic acid and therefore have the lL configuration. Investigations to resolve this problem are continuing.

2.3 Carbon-13 Magnetic Resonance Spectrum

The ¹³C nmr spectrum reproduced in Figure 3 was obtained from a D₂O/NaOD solution of calcium leucovorin on a Varian XL100 Spectrometer. The assignments on the TMS scale and referenced to dioxane are listed below:

<u>Assignment</u>	<u>Chemical Shift (ppm)</u>
C-2	93.7
C-4	102.0
C-6	0.0
C-7	- 23.3
$\begin{array}{c} \text{O} \\ \parallel \\ \text{-C-H} \end{array}$	98.2
C-9	- 25.0



LSJ-57

FIGURE 3 ^{13}C NMR OF CALCIUM LEUCOVORIN

<u>Assignment</u>	<u>Chemical Shift (ppm)</u>
C-4a or C-8a	25.2
C-8a or C-4a	86.5
C-1'	54.7
C-2', C-6'	62.4
C-3', C-5'	45.7
C-4'	84.8
C-7'	102.8
C- α	- 10.8
C- β	- 37.9
C- γ	- 32.3
α -COO ⁻	112.6
γ -COO ⁻	115.6

The above assignments are in reasonable agreement with those previously reported for pteridines.⁴

2.4 Ultraviolet Spectrum

Figure 4 represents the uv spectrum of calcium leucovorin in pH 7.0 phosphate buffer (0.10 M), $\lambda_{\max} = 286$ nm, $\lambda_{\min} = 243$ nm, and in 0.10N NaOH (pH 13), $\lambda_{\max} = 282$ nm, $\lambda_{\min} = 242$ nm. These spectral features are similar to those reported in the literature.⁵⁻¹² The most commonly reported absorptivities for leucovorin or folinic acid were obtained from solutions prepared in 0.1N NaOH; these values vary from $\sim 2.7 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ to $3.26 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.¹¹ Robinson¹² reports ϵ_{282} approximately 5% lower (at pH 8.4) than those in basic solution. The single absorption is due to contributions from p-amino-benzoylglutamate and the unsaturated portion of the pteridine ring. Loss of absorbance at shorter and longer wavelengths in basic solution (256 nm and 368 nm) is characteristic of reduced folates. Spectral data obtained from solutions prepared in acids do not reflect leucovorin absorbances because the compound is not stable. Leucovorin dehydrates under acidic conditions to produce anhydroleucovorin, N⁵,N¹⁰-methenyltetrahydrofolic acid, IV. This compound has been isolated in more than one form, depending on pH.¹³

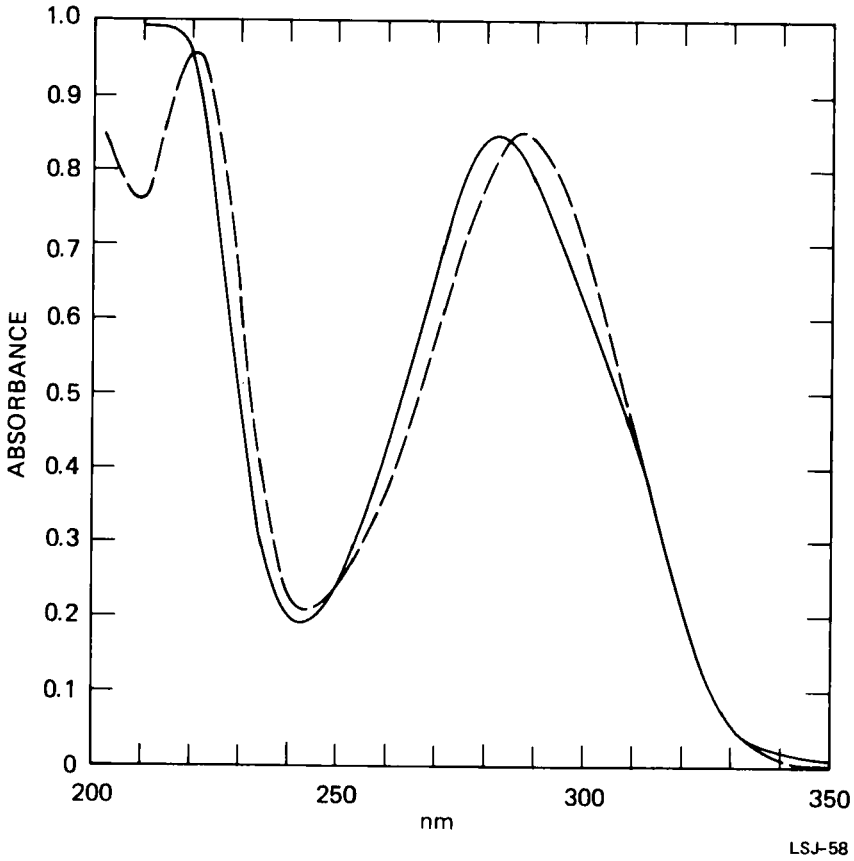


FIGURE 4 ULTRAVIOLET SPECTRUM OF CALCIUM LEUCOVORIN
0.1N NaOH ———
pH 7 Phosphate buffer — — —

2.5 Mass Spectrum

An electron impact mass spectrum of calcium leucovorin has not been obtained because the compound is not sufficiently volatile. It would be difficult to isolate the free acid without first dehydrating the compound. Due to its ionic nature, calcium leucovorin will not dissolve in common silylating reagents. Field desorption, another mass spectral technique, generally lends itself more to compounds like leucovorin. Indeed, this technique has been applied successfully to methotrexate and other folic acid analogs.¹⁴

2.6 X-ray Crystallographic Data

Crystallographic data have not been published for calcium leucovorin, but literature values do exist for the barium salt.⁷ Diffraction patterns could not be obtained on all samples although they had been recrystallized several times. Interplanar spacings are listed, but these numbers appear to change with the amount of moisture present in the sample.

2.7 Optical Rotation

$$\alpha]_{589}^{21^{\circ}} = + 14.3 \pm 0.4^{\circ} \text{ (c } 1, \text{ N/10 NaOH)}$$

$$\alpha]_{546}^{21^{\circ}} = + 17.9 \pm 0.4^{\circ} \text{ (c } 1, \text{ N/10 NaOH)}$$

$$\alpha]_{589}^{21^{\circ}} = + 14.9 \pm 0.4^{\circ} \text{ (c } 1, \text{ H}_2\text{O)}$$

$$\alpha]_{546}^{21^{\circ}} = + 18.8 \pm 0.4^{\circ} \text{ (c } 1, \text{ H}_2\text{O)}$$

The above values were obtained on an average lot of calcium leucovorin examined in this laboratory. Corrections were made for water content. Differences in rotation values may partly reflect solubility in the two solvents; the compound was more difficult to dissolve in N/10 NaOH. The H₂O rotation is close to the literature value: $\alpha]_D = + 14.26^{\circ}$ (c, 3.42 H₂O).²

2.8 Circular Dichroism

The following data were obtained on an average sample of calcium leucovorin:

<u>Solvent</u>	<u>λ_{\max} (nm)</u>	<u>$[\theta]$ (deg M⁻¹cm⁻¹)</u>
0.1N NaOH	282	3.89×10^3
H ₂ O	287	3.47×10^3

The molar ellipticities are calculated for leucovorin free acid and have been corrected for water content. These values may be quite different from those of folinic acid. Although a single value is reported for each solvent, both spectra contain more than one absorption band. Whether the extraneous absorptions are due to impurities or are actually due to leucovorin's absorption behavior is not known at this time.

2.9 Dissociation Constants

Three pKa values have been reported for leucovorin (free acid); they are 3.1, 4.8, and 10.4, as determined by electrometric titration.⁶ The first two values are attributed to the glutamyl carboxyls, and 10.4 is assigned to the hydroxyl group at the 4 position,⁸ by comparison to model compounds.

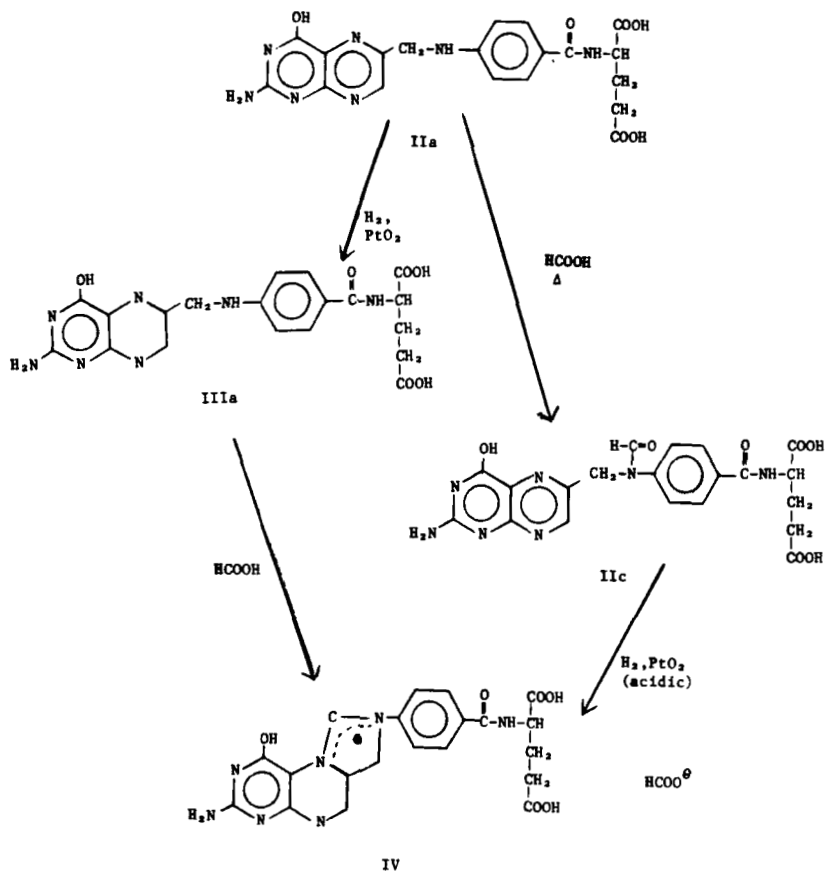
2.10 Solubility

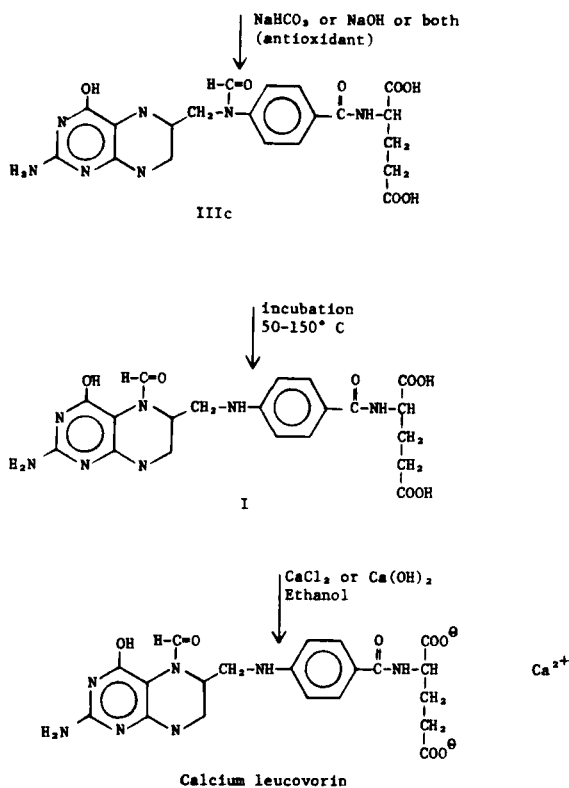
The ionic nature of calcium leucovorin severely restricts its solubility in common organic solvents (solubility in DMSO \ll 1 mg/ml). In water, the solubility is large (\sim 100 mg/ml), but in 0.1N NaOH it drops significantly ($<$ 20 mg/ml). Acid solubility is difficult to interpret since the solute may no longer be leucovorin.

3. Synthesis

With slight modifications in procedure, one basic synthesis of leucovorin and the subsequent isolation of its salts have remained unchanged for nearly thirty years. The method involves hydrogenation of folic acid (pteroylglutamic acid) in the presence of a platinum or palladium catalyst, as first described by O'Dell et al.¹⁵ This

reduction step is carried on concurrently or after¹⁶⁻¹⁹ initial formylation of folic acid in formic acid. The pH of the reaction mixture is adjusted with base in the presence of an antioxidant and heated (incubated 50-150° C with or without reduced pressure). Purification by column chromatography is followed by precipitation of the desired salt by addition of the appropriate cation and organic solvent. The calcium salt is precipitated from an aqueous solution by addition of CaCl_2 followed by ethanol.²⁰



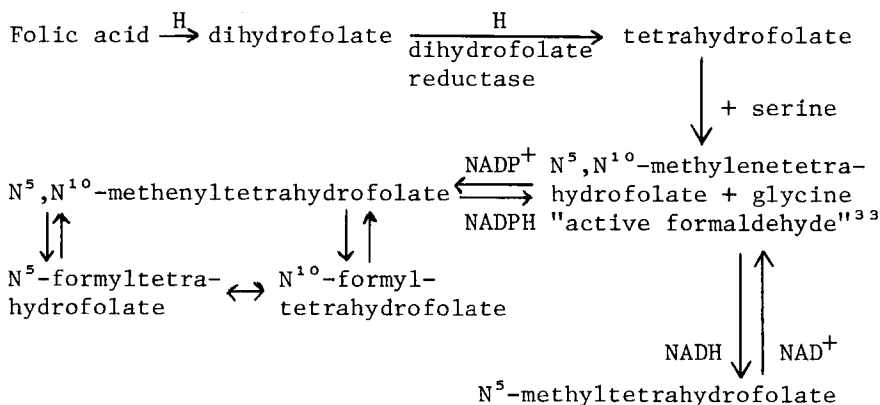


Early syntheses^{7,16} employed ascorbic acid as the anti-oxidant to protect the newly formed tetrahydro ring. But Roth et al.⁹ found that ascorbic acid had no effect on leucovorin yield (40-50%) as long as incubation was performed under anaerobic conditions. More recent syntheses have used 2-mercaptoethanol²¹ or a stream of nitrogen²² to protect the tetrahydro system.

Isolation of the active component is carried out chromatographically. Roth et al.⁹ used a magnesol (magnesium silicate) column to absorb colored impurities, followed by a Darco G-60 activated carbon column to eliminate sodium formate and inorganics. Elution with an alcohol/ammonia solvent was followed by rechromatographing in magnesol.

Flynn et al.⁶ used a potato starch column, elution with n-butanol/water/ethanol/glacial acetic acid, 100/50/40/0.4, followed by a Florisil column, treated with pH 5.5 acetate buffer, CaCl_2 and ascorbic acid and elution with distilled water. Subsequent recrystallizations were made from water. Other authors prefer ion-exchange cellulose chromatography. Zakrzewski and Sanson²¹ use DEAE cellulose in the OH^- form, eluting with a 2-mercaptoethanol/ammonia gradient. Beavon and Blair²² recommend 1-mm cellulose plates developed in n-propanol/ $\text{NH}_4\text{OH}/\text{H}_2\text{O}$, 200/1/99.

The chemical synthetic methods of obtaining leucovorin or its salts are discussed above, but of course the originally identified material came from biological synthesis of citrovorum factor in animal liver.¹ Early isolation techniques included electrolysis of liver concentrates in the presence of acetic acid.²³ Folinic acid synthesis has been reported in a variety of biological systems, including rat liver,²⁴ avian liver homogenates,^{25,26} microorganisms, (i.e., bacteria^{27,28,29} and virus³⁰) and plants.³¹ Factors affecting synthesis (e.g., temperature, pH, antioxidants) are discussed in several of these papers. In living systems, folinic acid can be synthesized ultimately from folic acid by reduction to tetrahydrofolic acid followed by addition of a 1-carbon fragment to the molecule ($\text{N}^5, \text{N}^{10}$ -methylenetetrahydrofolate, V). After a 2-step oxidation, the formyl group resides either at the N^5 or N^{10} position or as an equilibrium mixture. The essential reactions are summarized below:³²



Recently, the enzymatic formation of folinic acid has been utilized to synthesize radioactively labeled products.³⁴ The preparation of 5-formyl tetrahydrofolate, 9,3',5'-³H and 5-formyl-¹⁴C-tetrahydrofolate starts with tritiated folic acid, which is reduced to dihydrofolate, incubated in the presence of formaldehyde, dihydrofolate reductase, and NADPH, and finally incubated with 5,10-methylenetetrahydrofolate dihydrogenase. The product, N⁵,N¹⁰-methenyltetrahydrofolate (with ascorbic acid) was adjusted to neutral pH, autoclaved, and stored at -20° C prior to column purification on DEAE and G-15 Sephadex. These labeled products are the biologically active diastereomers, and they are used to study the metabolism of folinic acid in cells, tissues, and animals.

4. Stability

4.1 Bulk

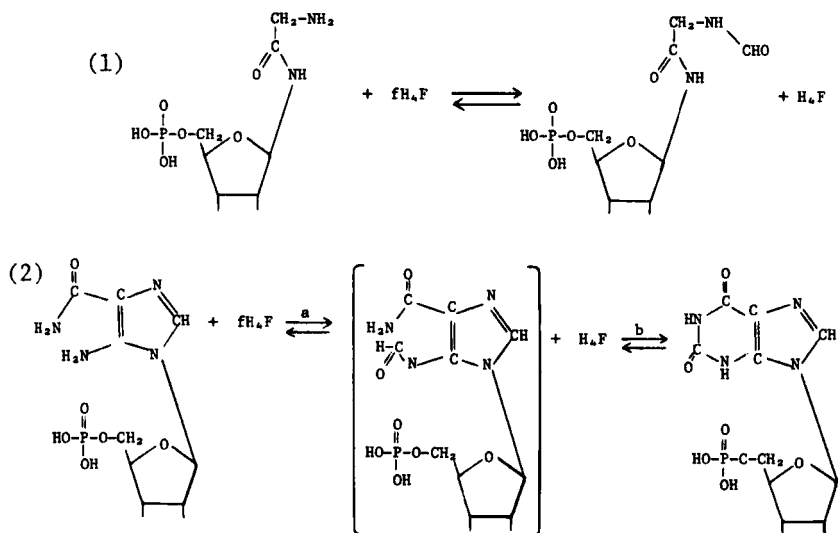
Calcium leucovorin is stable in bulk form after four weeks' storage at 60° C. The stability was monitored by HPLC in this laboratory, using the conditions discussed in Section 6.6.4.

4.2 Solution

Citrovorum factor has been reported to be stable under severe alkaline conditions by Broquist et al.³⁵ and earlier by Lyman and Prescott.³⁶ Steam-heating for 30 minutes in 0.2N NaOH did not decompose the compound. Alkaline stability is not surprising when one considers that many of the synthetic procedures require autoclaving at high pH as a final step in converting anhydroleucovorin. Investigations conducted in this laboratory in 0.1N NaOH (pH ~ 13) and in deionized water (pH ~ 6 show leucovorin to be stable at room temperature and under laboratory illumination for at least 24 hours (concentration 1 mg/ml). Stability was monitored by high-pressure liquid chromatography using conditions found in Section 6.6.4. As stated previously, leucovorin is not stable under acidic conditions.¹³ Depending on the pH, different forms of anhydroleucovorin have been isolated: pH ≤ 1.3 isoleucovorin chloride; pH = 2 anhydroleucovorin A; pH = 4 (hot) anhydroleucovorin B. All three forms convert back to leucovorin when dissolved in NaOH under anaerobic conditions.

5. Metabolism

Citrovorum factor functions (primarily) as a 1-carbon donor in the synthesis of serine from glycine.³⁷⁻⁴⁰ The N⁵ formyl group is incorporated from the 5,10 methylene compound.^{33,39} Silverman et al.⁴¹ have shown that the citrovorum factor, when in the presence of a large excess of glutamic acid and a hog liver enzyme, loses its formyl group to form the N-formylglutamic acid and tetrahydrofolic acid. These authors dismiss the possible formation of 10fH₄F or 5,10-methenyl H₄F in their reactions. However, Peters and Greenberg⁴² reported separating an enzyme from sheep liver, citrovorum factor cyclohydrazase, which converted 5fH₄F to a compound much like, but not identical to, 5,10-methenyl H₄F. Anhydroleucovorin, the 5,10-methenyl H₄F compound, exists in equilibrium with the 5 and 10 formyl compounds (see biosynthetic scheme). The latter, 10fH₄F, has been reported to donate its formyl group to methionine, which is associated with an esterified species A transfer RNA, which in turn continues in the synthesis of proteins.⁴³ In the presence of appropriate enzymes, formyltetrahydrofolates donate formyl groups for purine synthesis⁴⁴:



Equation 1 is catalyzed by glycinamide ribotide (GAR) transformylase and Equation 2 is catalyzed by aminoimidazole-carboxamide ribotide (AICAR) transformylase.

In humans and rats, early investigations showed that large doses of folic acid resulted in increased excretion of a substance that stimulated the growth of P. cerevisiae and that was presumed to be citrovorum factor.⁴⁵ This response is now also associated with other reduced folates.

Recently a great deal of effort has been spent in studying the metabolism of leucovorin in vivo. This emphasis was prompted by the chemical stability of this folate and by the observation of a reduction in toxicity of methotrexate when it was given in conjunction with leucovorin. Folinic acid is found in human liver, but it is not the major circulating folate, which is 5-methyltetrahydrofolate. Most of the folates that occur naturally in man are polyglutamates,⁴⁶ although they are transported as monoglutamates.⁴⁷

Several groups investigating human metabolism of folinic acid have used a differential microbiological method for determining folates in bile, serum, and urine.^{47,49} Using three microorganisms that respond to different folates, they deduced the identity of some metabolic products. Generally, L. casei responds to all monoglutamates, S. faecalis also measures monoglutamates except 5mH₄F, and P. cerevisiae growth quantitates tetrahydrofolates, again excluding 5mH₄F. Pratt and Cooper⁴⁸ showed a large increase in L. casei response and a less pronounced increase in S. faecalis growth in bile and plasma after oral administration of leucovorin to patients. Little, if any, increase was noticed in P. cerevisiae. From these observations they concluded that 5fH₄F is rapidly metabolized to 5mH₄F, although a very small amount may be absorbed as the intact 5-formyl compound. Perry and Chanarin⁴⁹ found similar results in urine samples. With the synthesis of the doubly labeled radioactive compound,³⁴ 5-formyl-¹⁴C-tetrahydrofolate-³H, investigators have been able to trace the metabolic products when the drug is administered orally or intravenously.⁵⁰

Nixon and Bertino have done an extensive study of folinic acid metabolism in human subjects given the drug both ways. When given orally, after 75 minutes the majority (90%) of ^3H in serum was found in $5\text{mH}_4\text{F}$, with 20% of ^{14}C associated with the same compound; 8 to 9% of each label was found as $10\text{fH}_4\text{F}$ or 5,10-methenyl H_4F . Due to the labile nature of the formyl group, 70% of the ^{14}C was not absorbed on Sephadex as folate under the chromatographic conditions used, and the authors presumed that radioactivity had been incorporated into amino acids. Virtually no radioactive $5\text{fH}_4\text{F}$ was found. However, the radioactivity associated with urine (0-1 hour after administration) was $\sim 40\%$ ^3H -labeled $5\text{fH}_4\text{F}$. Less than 20% of the remaining ^3H was identified as $5\text{mH}_4\text{F}$ or p-aminobenzoylglutamate, less than 40% as $10\text{fH}_4\text{F}$ or 5,10-methenyl H_4F . The ^{14}C was excreted as a mixture of the three folates. At longer time intervals, these proportions varied. Ninety minutes after injection, 60% ^3H was found as $5\text{mH}_4\text{F}$, 40% of radioactivity as $5\text{fH}_4\text{F}$, and 40% ^{14}C was no longer associated with serum folate. Urine showed most radioactivity as $5\text{fH}_4\text{F}$ initially, and several hours later a rise was seen in labeled $10\text{fH}_4\text{F}$ and/or 5,10-methenyl H_4F . From this series of studies, the authors concluded that most ^{14}C was removed from circulating folates, most of the ^3H was associated with $5\text{mH}_4\text{F}$, most of the drug was absorbed by tissues, and the majority of $5\text{fH}_4\text{F}$ was converted to $5\text{mH}_4\text{F}$. Equally important was the site of metabolism. Since the $5\text{f} \rightarrow 5\text{m}$ conversion was more rapid in patients receiving oral administration, the authors felt that the reaction took place in the intestine, a view supported by others.⁵¹ The voided folates seemed to be $10\text{fH}_4\text{F}$ or 5,10-methenyl H_4F . Another interesting suggestion was that $5\text{mH}_4\text{F}$ was conserved over $5\text{fH}_4\text{F}$ by the kidneys.

Although leucovorin is rapidly absorbed and metabolized in the intestine, its metabolic product, $5\text{mH}_4\text{F}$, is concentrated in cerebrospinal fluid (CSF). In a study with dogs, Levitt et al.⁵² found that injected labeled folates disappeared from serum and appeared as $5\text{mH}_4\text{F}$ in CSF following first-order kinetics. With doubly labeled $5\text{fH}_4\text{F}$, the authors were able to trace the ^{14}C formyl moiety to serine or methionine and the ^3H portion to $5\text{mH}_4\text{F}$. The uptake of drug occurred in two steps:

- a) equilibration in extracellular fluid with nonlabeled

compound,⁵³ followed by b) cellular uptake and metabolism. Early papers reported a natural concentration of folate in CSF that was three times the equilibrium concentration in serum. This could indicate the importance of folates in neural metabolism, and there have been some reports regarding the therapeutic treatment of neuropsychiatric disorders with folates.

The recent interest in leucovorin is due to its ability to reduce methotrexate (MTX) toxicity when both are administered to cancer patients. Early studies⁵⁴ in mice showed that most benefit occurred when leucovorin was given 12-24 hours after MTX infusion. The delay in administering leucovorin reduced the toxic effects of MTX without reducing the latter's antitumor activity. Therefore, an enhancement of MTX effect can be produced by larger dosages without the toxicity associated with these amounts. When given concurrently, leucovorin reduced MTX toxicity at the expense of tumor activity. However, recent work⁵⁵ has shown some advantage in concurrent administration. In the case of MTX-sensitive cells, "folinic acid protection" increases the cell survival percentage. Rescue procedure, or leucovorin given after a delay period, was most effective (measured in percentage of cell survival) in treating MTX-resistant cells. The therapeutic effect of leucovorin lies in an earlier resumption of DNA synthesis than if MTX were administered alone.⁵⁶ Nahas et al.⁵⁷ attribute this resumption to several possible actions of leucovorin (as studied in L1210 leukemia cells): a) efflux of MTX is increased in the presence of 5fH₄F; b) 5fH₄F could deblock dihydrofolate reductase bound by MTX by being a potential supply of dihydrofolate; c) the intact drug could possibly displace some of the MTX; and d) 5fH₄F competes with MTX for cellular uptake. Cellular uptake seems to be enhanced by methyl or formyl groups at N⁵ on reduced folates, by amino substitutions on C₄ of oxidized folates, and by terminal glutamates.⁴⁷ Thus 5fH₄F, 5mH₄F, and MTX appear to use the same carrier-mediated transport into cells, a system used by folinic acid to a lesser extent. Another author⁵⁸ describes the transport of folates across membranes as an exchange phenomenon because although leucovorin competes with MTX for cell uptake, it can also stimulate influx of the latter if the cells are already

preloaded with folinic acid. The influx is noted even in cases where dihydrofolate reductase has been inactivated by previous MTX dosage.

6. Methods of Analysis

6.1 Elemental Analysis

Below are listed elemental analysis results obtained from a typical sample of calcium leucovorin:

<u>Element</u>	<u>% Theoretical</u>	<u>% Found*</u>
C	46.96	46.87
H	4.14	4.11
N	19.17	19.07
Ca	7.84	6.94 [†] , 7.71 [‡]

* Corrected for 10.4% H₂O.

[†] Found by atomic absorption.

[‡] Found by sulfate ash.

The higher calcium value obtained by the ash method is no doubt due to the presence of sodium (the counter ion for acetate commonly found in some lots).

6.2 Equivalent Weight Determination

Because synthetic products are isolated as the barium or, more frequently, the calcium salt of leucovorin, common acid-base titrations are not reported. If this type of titration or one in which the cation is exchanged were feasible, the results would require careful interpretation because impurities containing the glutamic acid moiety would respond similarly to leucovorin when the carboxyl groups are being analyzed.

6.3 Biological Assay

6.3.1 Microbiological Assay

Several microbiological procedures for assaying CF have been described in the literature. In general, these methods are designed to measure CF content in a biological fluid or specimen. They all make use of the growth promotion of L. citrovorum 8081, or P. cerevisiae, as it has been renamed. Before CF was isolated

or leucovorin was synthesized, Sauberlich and Baumann¹ measured enhanced growth of the microorganism in the presence of various biological extracts, especially liver concentrates. Quantitation came from turbidometric readings and/or NaOH titration of acid produced. Their results are reported in terms of citrovorum units that corresponded to the half-optimal growth in a standard reticulogen sample. The authors realized the limits of their assay method since folic acid additions caused enhanced growth after an initial lag. Winsten and Eigen⁵⁹ tried to improve the method by chromatographing the samples on paper before testing for P. cerevisiae growth, but they, too, found more than a single active component. The basis of this particular technique is still being used. Cooperman⁶⁰ refined the procedure somewhat, but continued to measure turbidity. Folic acid interference is minimized by incubation for short periods. Quantitation is in terms of mg/ml, using commercial samples of leucovorin as standards. In one recently published method,⁶¹ paper discs are impregnated with the test solutions and CF content is found by manually measuring L. citrovorum ATCC 8081 growth on agar plates. The advantage of this method is that CF can be quantitated in solution with a large excess of amethopterin because the microorganisms are resistant to the latter. Statistical variation on this method is $\sim 10\%$.

6.3.2 Enzyme Assay

Silverman et al.⁴¹ purified a hog liver enzyme that catalyzed the transfer of the formyl group of CF to glutamic acid. If not protected from oxidation, the tetrahydrofolic acid formed would spontaneously decompose to p-aminobenzoylglutamate and pteridine. Arylamine formation could be monitored by the Bratton-Marshall method. This method gives a better indication of enzyme activity than CF purity. The technique developed by Peters and Greenberg,⁴² later refined by Greenberg,⁶² is perhaps more straightforward. The test solutions are mixed with ATP, MgSO₄, and enzyme isolated from sheep liver in a buffered aqueous solvent. Absorbance at 343 nm is read in a spectrophotometer at 30° C. The 5,10-methenyltetrahydrofolate being formed should give a direct measure of the CF or leucovorin present.

The above biological assays are intended to measure CF in biological sources rather than to directly measure leucovorin or CF purity. In fact, commercial samples of leucovorin are usually used to determine standard curves.

6.4 Polarographic Assay

Leucovorin, since it is totally reduced, is polarographically inert in a pH 9 buffered solution.⁶³ After acid treatment, three polarographic waves are generated, corresponding to an anodic oxidation of a tetrahydro compound and two cathodic reductions of unreduced pteridines; presumably at least one of these three is a dihydro species. Polarography is useful as a technique in structural elucidation, but analytical data would be difficult to obtain from an acid-treated solution containing several species, each with its own polarographic behavior.

6.5 Spectrophotometric Analysis

6.5.1 Fluorometric Analysis

In 1957, Duggan et al.⁶⁴ reported that maximum natural fluorescence of folinic acid occurred at pH 7, with excitation at 370 nm and emission at 460 nm. A concentration of 0.15 $\mu\text{g/ml}$ gave a fluorescence of 10% full-scale deflection at maximum instrumental sensitivity. These authors explored analyzing folinic acid in the presence of folic acid and found that excitation at 290 nm effectively shifted the emission band of the compound of interest to 370 nm, thus enabling analysis of a mixture. A later paper⁶⁵ reported a fluorescence maximum for leucovorin at 365 nm when excited at 314 nm in a pH 7 solution; the concentration was 5×10^{-5} M. Variation between these data and other values was attributed to sample impurity, pH of solution, and quenching. The authors made an attempt to correlate structure and fluorescence of reduced folates. Similarity between tested compounds and *p*-aminobenzoyl-glutamate lead them to conclude that this portion of the molecule is responsible for maxima at 360-425 nm when excited at 300-320 nm. They suggested that intensity differences may arise from various substitutions on the tetrahydropteridine moiety.

In 1964, Netrawali et al.⁶⁶ described fluorescence measurements of calcium leucovorin in NaHCO_3 solution after paper chromatography and reaction with acidic orcinol. The gray-blue fluorescent species formed on the developed paper-gram was very sensitive for leucovorin, showing a detection limit of 0.1 μg . In the eluting solution, however, the required concentration increased to 0.5 μg . Recovery by this method was $100 \pm 15\%$, and results obtained from fluorescence were lower by 10 to 20% than those obtained by microbiological methods.

6.5.2 Ultraviolet/Visible Analysis

Ultraviolet spectroscopy does not lend itself to leucovorin analysis for two reasons. First, because commercial samples are frequently contaminated with uv-absorbing impurities, a reliable molar absorptivity has not been determined for leucovorin. Recently, in this laboratory a value of $3.09 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ was derived from thorough analysis of a relatively pure sample. This value is in reasonable agreement with that of Zakrzewski and Sanson.²¹

Second, leucovorin is known to dehydrate under acidic conditions to form anhydroleucovorin, 5,10-methenyl H_4F , which absorbs at 352-353 nm. In the absence of interfering species,* leucovorin may be analyzed by acidification with 0.1N HCl followed by uv measurement after 1.5-2.0 hours. Purity may be determined relative to a sample of known purity or relative to literature values:
 $\epsilon_{352} = 2.39\text{-}2.41 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.²¹

* Absorption interferences would arise from compounds other than leucovorin that convert to anhydroleucovorin at acidic pH. An example of such a compound is 10f H_4F , although it is not expected to contribute to absorption due to its instability. However, 10-formyl-7,8-dihydrofolate is a possible contaminant. Additional absorption could also result from unsaturated pteridines.

6.6 Chromatography

6.6.1 Paper Chromatography

Both paper and thin-layer chromatography serve as separation techniques before quantitation of active components. Paper chromatography has been used by many early investigators to separate biological samples before detecting by microbiological assay, the bioautographic method. Winsten and Eigen⁵⁹ used 2,4,6-trimethylpyridine to develop papergrams on Whatman No. 1 or No. 4. Colón²⁹ developed his chromatograms in isoamyl alcohol:5% dibasic sodium phosphate, 1:2. Netrawali et al.⁶⁶ used ethanol:n-butanol:water:25% NH₃, 50:15:35:5, on Whatman No. 1 and detected leucovorin by forming a fluorescent orcinol derivative. The mobile phases in these systems are neutral to basic due to the acid instability of leucovorin.

6.6.2 Thin-layer Chromatography

Thin-layer chromatography on cellulose has been used as an intermediate clean-up procedure and final isolation technique in the synthesis of leucovorin.²² A cation exchange/cellulose support was prepared by Copenhaver and O'Brien,⁶⁷ and the mobile phase was 15% Na₂HPO₄•12H₂O (pH 8.5) containing 0.1 M mercaptoethanol. Unfortunately, this system did not separate 5f and 10fH₄F from each other or from 5,10-methenyl H₄F. Separation of the former two from the latter was achieved with 1% Na₂HPO₄•12H₂O. Leucovorin was detected with a 6N HCl/ZnCl₂/sodium citrate spray.

Brown et al.⁶⁸ have developed a cellulose plate with a fluorescent indicator. Compounds are developed in 3.0% (w/v) NH₄Cl and detected by fluorescence quenching. These authors also use 0.5% mercaptoethanol in their mobile phase, but this is only to prevent oxidation of the labile reduced pteridines, which are not adequately protected by substitution at the N⁵ position. Since neutral or alkaline solutions of leucovorin are relatively stable in air, this precaution may not be required for routine assay.

6.6.3 Column Chromatography

Leucovorin has been chromatographed on columns of different packing materials since it was first synthesized. Early investigators used magnesium silicate and activated charcoal,⁹ starch,⁶ and--more recently--DEAE cellulose²¹ in their purification and isolation of leucovorin. Column techniques have been coupled with various detection methods to identify and quantitate CF and other folates in naturally occurring products. Noronha and Silverman⁶⁹ used L. casei, P. cerevisiae, and S. faecalis to detect folates in chicken liver extracts chromatographed on DEAE cellulose. A similar method was used by Rohringer et al.⁷⁰ to monitor folates in healthy and infected wheat leaves. An earlier publication⁷¹ reported anion-exchange chromatography on Dowex-1-chloride using uv absorption of collected fractions for detection. Due to its instability in acid, leucovorin was eluted with NaCl rather than HCl, which is commonly used for other folates. This method was limited to synthetic products rather than biological mixtures. The authors felt that sensitivity could be gained by microbiological detection.

More recently, attention has been placed on gel permeation techniques. Sephadex G-15 and G-25 columns coupled with uv detectors were used to separate folates found in rat kidney extracts.⁷² Kás and Cerná⁷³ used Sephadex G-10 to separate folates in cow's milk, utilizing spectroscopic and microbiological detection. Nixon and Bertino⁷⁴ used fluorescence ratios and markers on DEAE Sephadex A-25 to separate folate coenzymes. In separating folates from biological sources, several authors treated their samples with the appropriate conjugase to hydrolyze polyglutamates. Radioactivity measurement has been used to detect doubly labeled 5fH₄F and its metabolic products.⁵⁰

6.6.4 High-Pressure Liquid Chromatography

A natural extension of column chromatography is high-pressure liquid chromatography, which combines separation, detection, and quantitation (and isolation in the case of preparatory work). Perhaps the single

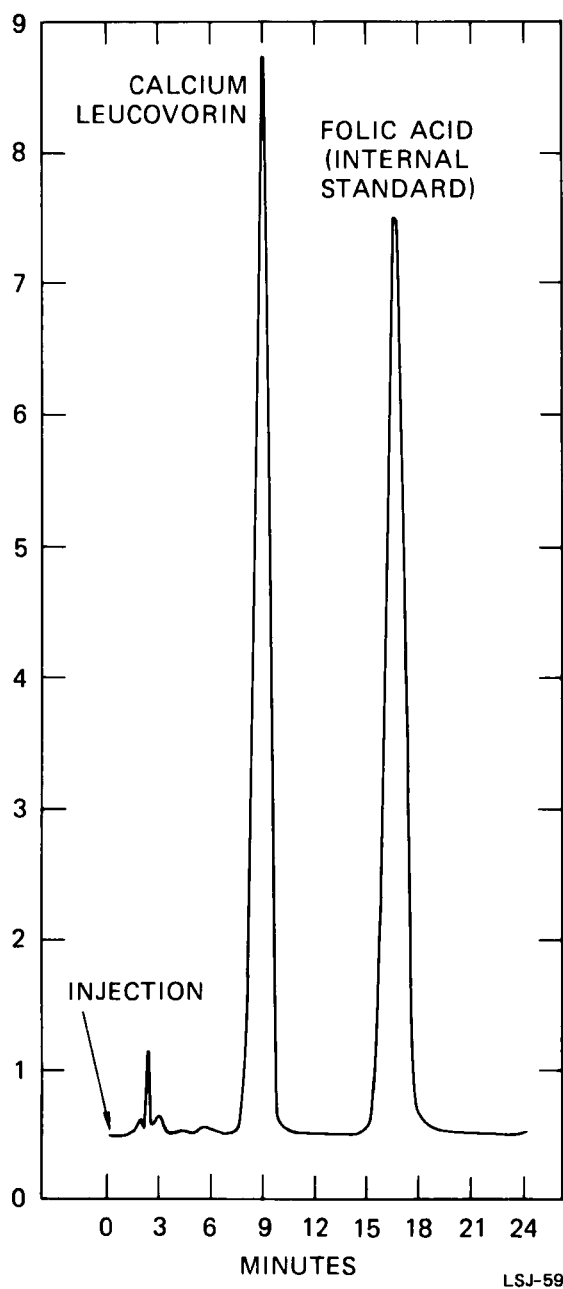


FIGURE 5 HIGH PRESSURE LIQUID CHROMATOGRAM OF CALCIUM LEUCOVORIN

most attractive feature of this technique is speed, once the chromatographic conditions have been determined. Various modes are available, but folates have been restricted to ion exchange or partitioning due to limited solubility in organic solvents. Detection is usually by uv absorption, although in the case of leucovorin, sensitivity is increased several orders of magnitude by electrochemical detection. HPLC has been used routinely in this laboratory since 1973, and among the compounds analyzed are folates.⁷⁵ The most versatile system that these authors have found is a chemically bonded C₁₈ column with 0.1 M KH₂PO₄ (pH adjusted to 4.0) containing varying amounts of methanol (see Figure 5). An earlier system using the same reverse phase C₁₈ column with tris buffer, 2-amino-2-(hydroxymethyl)-1,3-propanediol at pH 6.7, was abandoned in favor of the above phosphate eluent which better resolves contaminants from the major component. A similar set of conditions was utilized by Reif et al.⁷⁶ to analyze folic acid, using leucovorin as an internal standard. Other authors⁷⁷ have used similar conditions to study extensively the identity of possible leucovorin contaminants. In addition to these partition/ionization suppression methods, anion exchange hplc has been used to separate CF from other naturally occurring folates,⁷⁸ and some correlation has been made between the number of glutamyl residues and retention time.

6.6.5 Affinity Chromatography

Although affinity chromatography has not been used directly as an analytical method, it may be modified in the future to produce a viable technique. Leucovorin has been used as an effective spacer in obtaining active samples of dihydrofolate reductase.⁷⁹ If the enzyme could be immobilized without losing its activity, perhaps it could be used to separate folates.

6.7 Radioassay

Recently, radioassay methods have been refined to measure folates in biological samples. These techniques use radioactively labeled folates and competitive protein binding.⁸⁰ Johnson et al.⁸¹ compare this method with traditional microbiological assay with L. casei.

Although this method was designed for folates in general, differential microbiological testing could give an indication of reduced folate concentration.

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METHIMAZOLE

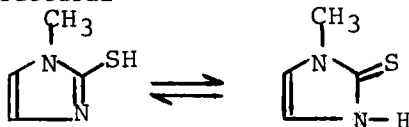
Hassan Y. Aboul-Enein and A. A. Al-Badr

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METHIMAZOLE

1. Description1.1 Nomenclature1.11 Chemical Names

1,3-dihydro-1-methyl, 2-H-Imidazole-2-thione.
 1-methylimidazole-2-thiol
 1-methyl-2-thioimidazole
 1-methyl-2-mercaptoimidazole

1.12 Generic Names Methimazole, thiamazole1.13 Trade Names Thiamethazole, Bazolan, Dananti-zol, Faristan, Frentorox, Mercazole, Metazole, Tapazole, Thacapazol, Thycapazol, Strumazol, Metothyrene.1.2 Formulae1.21 Empirical $C_4H_6N_2S$ 1.22 Structural1.3 Molecular weight 114.171.4 Elemental composition

C 42.08%, H 5.30%, N 24.54%, S 28.09%

1.5 Appearance, color, odor

White to pale buff, crystalline powder, having a faint characteristic odor. Its solution is practically neutral to litmus.

2. Physical properties2.1 Crystal properties2.11 Crystallinity

No detailed studies on the crystal structure of methimazole is reported in the literature. Methimazole can be microscopically identified using Kofler's method, the occurrence of polymorphous modification is indicated in methimazole.

2.12 Melting point

USP XIX (1) specifies a melting range for methimazole between 144 and 147°.

Leaflets from alcohol m.p. 146-148° (2), b.p. 280° (some decomposition).

2.2 Dipole Moments

The dipole moment of methimazole was determined in benzene and 1,4 dioxane solution at 25° (3) and reported to be 4.74 D and 5.53 respectively. The results were discussed in terms of tautomerism and molecular conformations.

2.3 Solubility

Freely soluble in water, in alcohol and in chloroform, slightly soluble in ether, petroleum ether and benzene.

2.4 Identification

The following tests are cited from USP XIX (1) :-

- a) The infrared absorption spectrum of a potassium bromide dispersion of it exhibits maxima only at the same wavelength as that of a similar preparation of USP methimazole Reference Standard.
- b) Mercuric chloride TS produces in a solution (1 in 200) a white precipitate, but no precipitation is produced by trinitrophenol TS. The solution is coloured intensely blue by molybdo-phosphotungstate TS.
- c) Methimazole can be identified by forming crystals of with gold bromide/HCl solution. Plates, often in crosses (sensitivity : 1 in 1000); potassium tri-iodide solution - bunches of rods or needles (sensitivity 1 in 1000) Fig. 1.

2.5 Spectral Properties

2.51 Ultraviolet spectrum

Methimazole in 0.1N sulfuric acid shows maxima at 211 nm (E 1%, 1 cm 593) and 251.5 nm (E 1%, 1 cm 1528). In neutral aqueous solution, methimazole absorbs ultraviolet radiation at 251.5 nm (Fig. 2). Hayden et al (5) published a report on the relation between the spectra and structure of methimazole and some related compounds. The replacement of an oxygen atom at C₂ by sulfur causes a big shift to longer wavelength with increased absorption(6).

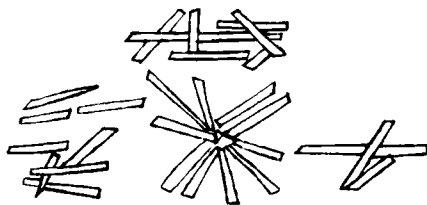


Fig. 1 - Methimazole - KI/I_2 crystals.

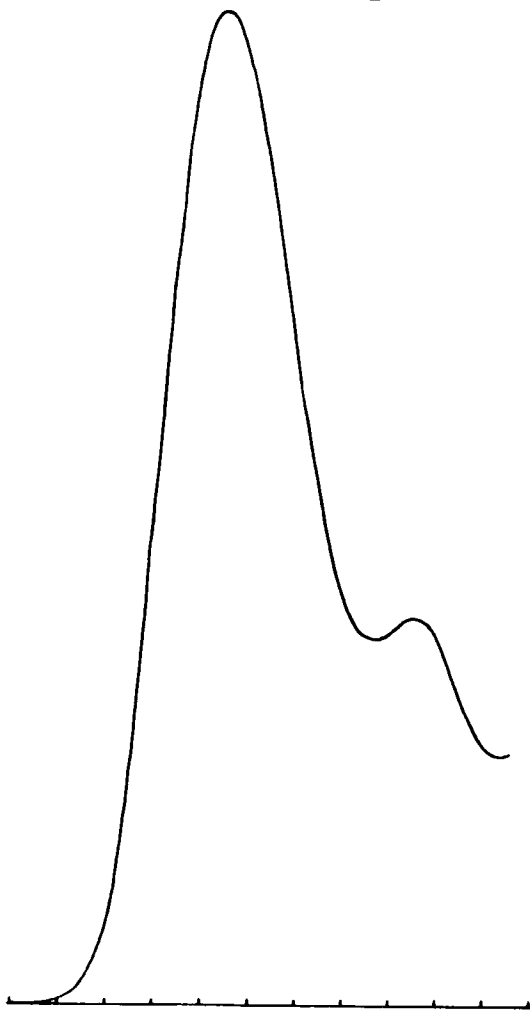


Fig. 2 - Ultraviolet spectrum of Methimazole in methanol.

2.52 Infrared spectrum

The infrared spectrum of methimazole is shown in Fig. 3. The spectrum was obtained from Nujol mull.

The structural assignments have been correlated with the following band frequencies:

Frequency (Cm^{-1})	Assignment
2500 - 2450 broad weak	- SH
1580	C = N aromatic

Other fingerprint bands characteristic to methimazole are at 1466, 1570 and 1271 Cm^{-1} as shown in Figure 4.

Further information with regards to the infrared spectra of methimazole is given in several references (4,5,7).

2.53 Nuclear Magnetic Resonance Spectrum

A typical NMR spectrum of methimazole is shown in Fig. 5. The sample was dissolved in CDCl_3 . The spectrum was determined on a Varian T-60 A NMR spectrometer with TMS as the internal standard. The following structural assignments have been made for Fig. 5.

Chemical Shift (δ)	Assignment
Singlet at 3.63	N-CH_3
Singlet at 6.70	aromatic H_4 and H_5 of the imidazole ring system.

Further information concerning the NMR spectrum of methimazole can be obtained from Sadtler NMR catalog (8) and also from CRC Atlas of spectral data (7) and Aldrich NMR catalog (9).

2.54 Mass Spectrum and Fragmentometry

The mass spectrum of methimazole obtained by electron impact ionization shows a pronounced molecular ion M^+ at m/e 114 (Fig. 6).

Bowie *et al* (10) studied the mass spectra of several imidazoles and methimazole was included. They discussed the fragmentation

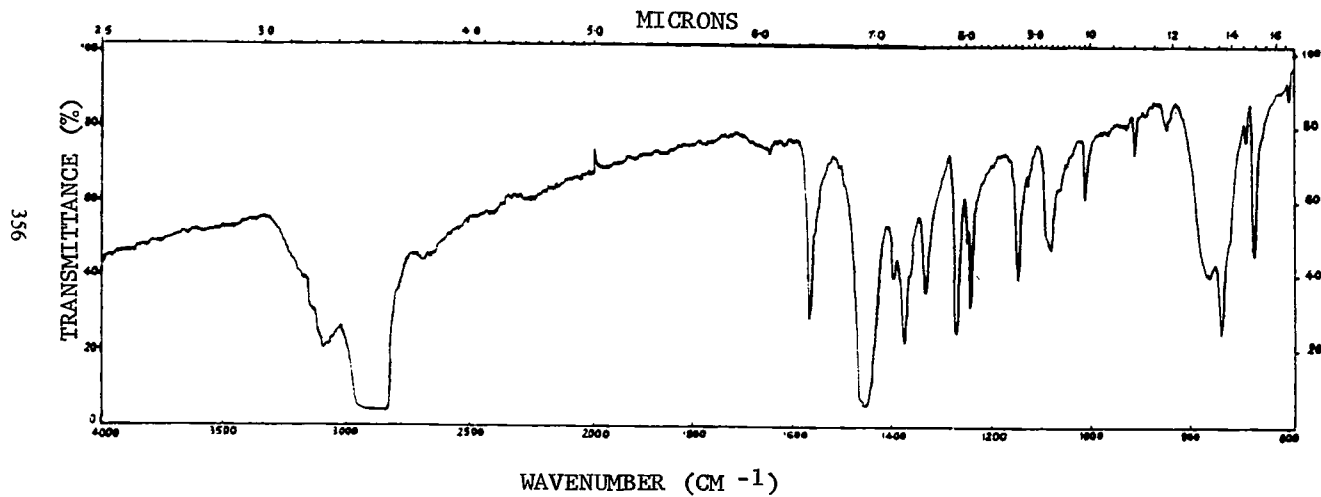


Fig. 3 - Infrared spectrum of methimazole in Nujol mull.

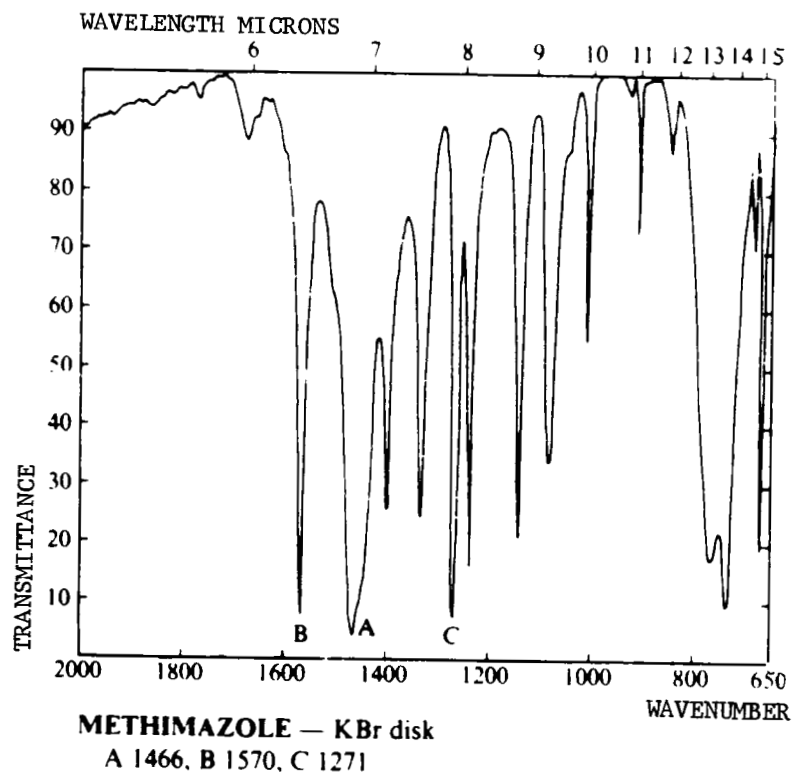


Fig. 4 - Fingerprints bands of methimazole, in KBr.

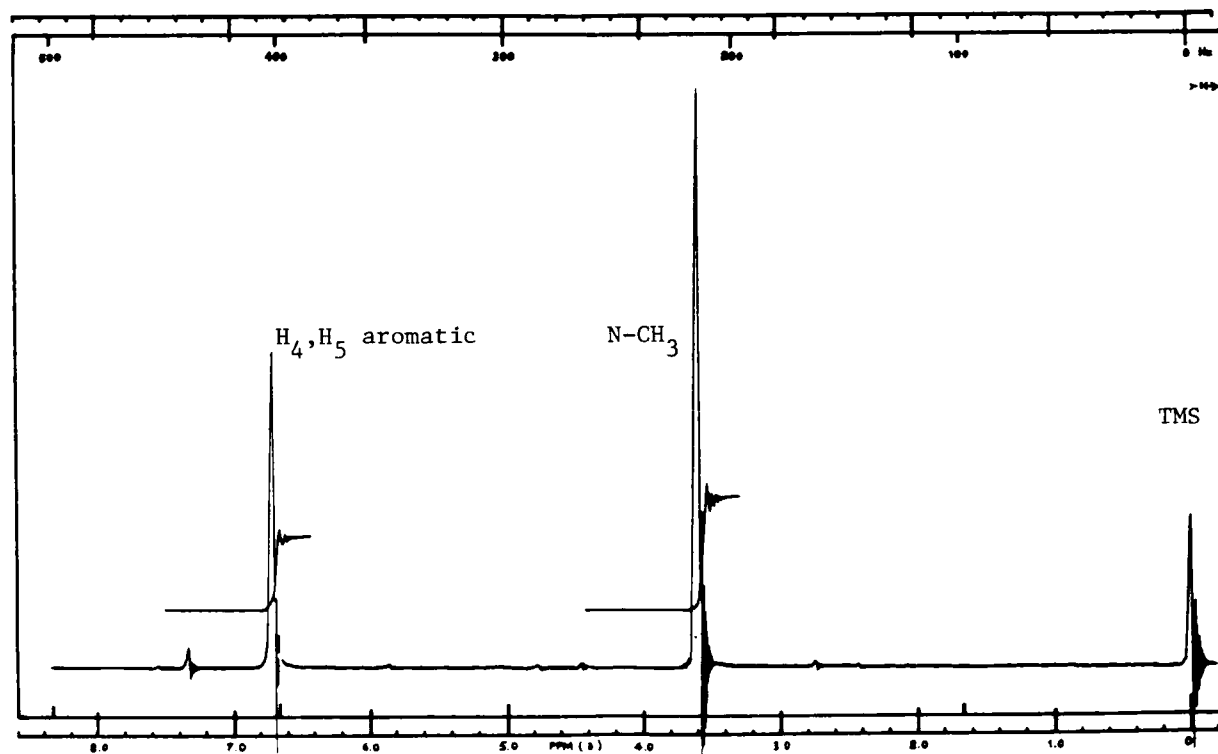


Fig. 5 - NMR spectrum of methimazole in CDCl_3 containing TMS as internal standard.

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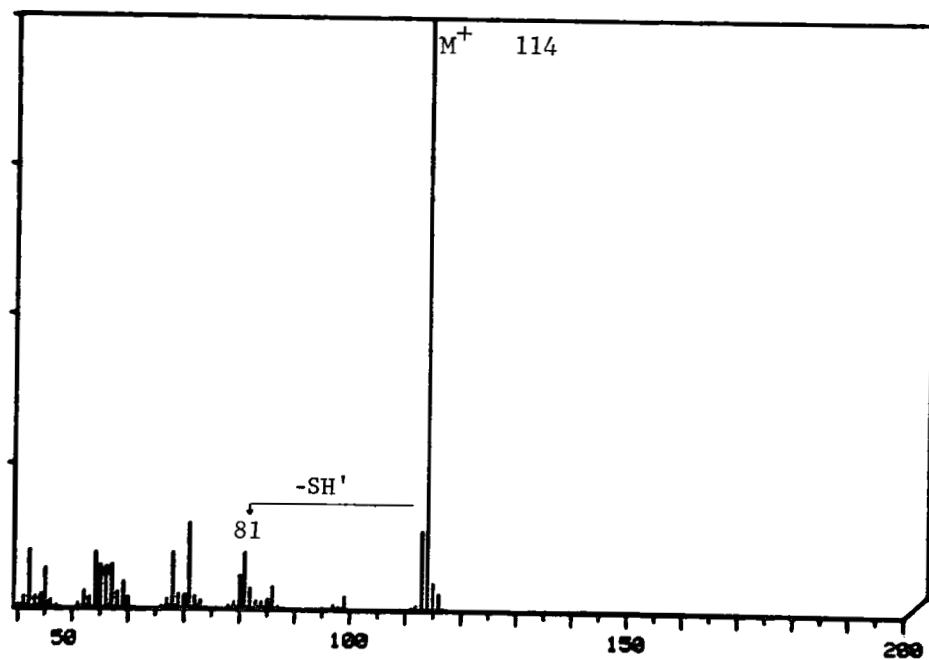
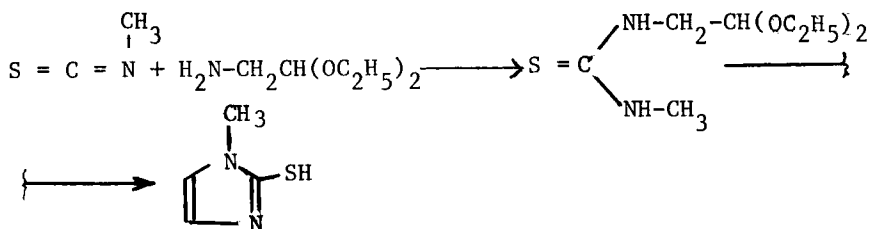


Fig. 6 - Mass Spectrum of Methimazole (EI).

patterns and modes which is substantiated by deuterium labelling, exact mass measurements and appropriate metastable ions. Skeletal rearrangements are rare in the imidazole ring system.

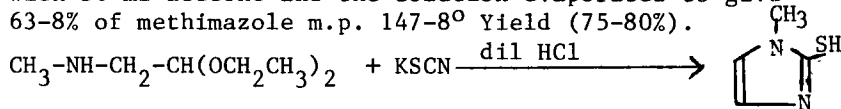
3. Synthesis

The method of preparation of methimazole illustrates the general synthesis of 2-mercaptoimidazoles by ring closure from α -amino-aldehydes or ketones and alkylisothiocyanates. This reaction gives 1-alkyl 2-imidazolethiols, and the simpler compounds unsubstituted in the 1-position can be made by the use of simple metallic thiocyanate in place of the alkyl compound. In the original synthesis of Wohl & Marckwald (*Ber.*, 22, 1354, 1889) of which a number of variations have since been introduced. Methimazole is made by condensing methyl isothiocyanate with amino-acetal, $\text{NH}_2\text{-CH}_2\text{CH(OC}_2\text{H}_5)_2$ (a convenient substitute for aminoacetaldehyde) and cyclizing the product by heating with acid. The 2-mercaptoimidazols can be alkyl-



ated or acylated on the sulphur or the second nitrogen atom or the whole SH group can be removed by oxidation with HNO_3 to give a simple imidazole.

Methimazole is also prepared (12,13) by the reaction of $\text{CH}_3\text{-NHCH}_2\text{-CH(O-Et)}_2$ with KSCN in the presence of dil. HCl. Hydrochloric acid (300 ml., 2N) was added gradually to a mixture of 72.5 gm of $\text{CH}_3\text{-NHCH}_2\text{-CH(O-Et)}_2$ and 56.4 gm of KSCN, the mixture kept 12 hours, evaporated to dryness, the residue refluxed 1 hour with 200 ml. of anhydrous acetone. The mixture filtered, the precipitate washed with 50 ml acetone and the solution evaporated to give 63-8% of methimazole m.p. $147\text{-}8^\circ$ Yield (75-80%).



4. Stability, Decomposition product and metal complexes

Methimazole is a relatively stable compound at room temperature. However, it is recommended that it should

be kept in a well-closed container protected from light and in a dry place.

Methimazole forms metal complexes with heavy metals e.g. Cu^{+2} , Al^{+3} , Fe^{+3} ions. Foye and Lo (14) studied the chelating properties of some imidazoles and other related heterocyclic thiones, and reported a relation between the metal binding strengths and their antimicrobial activity.

5. Metabolism

When 20 mg/kg of methimazole was administered i.p. or orally to rats, urinary methimazole glucuronides accounted for 36-48% of the dose in 24 hours. The only other urinary metabolite accounted for 10-20% and was not characterized. An additional 14-20% of methimazole was excreted unchanged in 24 hour urine. The bile contained methimazole glucuronide and two unidentified metabolites. One of which was the same as the unidentified urinary metabolites. Plasma proteins bound 5% of methimazole which had no affinity for any specific tissue. Methimazole had a much greater $\text{CHCl}_3/\text{H}_2\text{O}$ partition coefficient and H_2O solubility than did propylthiouracil. Between 77 and 95% of the methimazole was excreted in the urine and approximately 10% in the bile. Since fecal excretion was negligible; an enterohepatic circulation was present. The half life of urinary excretion was 5-7 hours regardless of the route of administration (15).

^{35}S - labelled methimazole given i.p. to rats accumulated in the thyroid gland where it was mainly oxidized to methimazole sulphate (16). Most of the label was excreted in the urine. The same results were found in man too (17). Pittman *et al* (18) showed that both the thyroid and adrenal glands had the highest organ to plasma ratios of the drug after four days of i.v. administration in rats. It was reported that methimazole in man, is more slowly absorbed and excreted than propylthiouracil. The plasma half-life in hours of methimazole ^{35}S was 2 or 5 times that of labelled propylthiouracil. The blood radio activity curve after the oral administration of carbimazole - ^{35}S was very similar to that of methimazole. It was suggested by Alexander *et al* that the renal function may have a more important influence on the biological half-life of the drug than the thyroid status (19). However, the identification of methimazole metabolites still need more investigation.

6. Methods of analysis

6.1. Titrimetric methods

6.11 Aqueous

Several titrimetric methods were developed for the analysis of methimazole

i) Silver nitrate method (1)

a) for the standard methimazole

Dissolve about 250 mg of methimazole, accurately weighed, in 75 ml of water. Add from a buret 15 ml of 0.1 N NaOH, mix and add with agitation, about 30 ml of 0.1 N AgNO₃. Add 1 ml of bromothymol blue TS, and continue the titration with the 0.1 N NaOH until a permanent, blue green color is produced. Each ml. of 0.1N NaOH is equivalent to 11.42 mg of C₄H₆N₂S.

b) For methimazole tablets

Weigh and finely powder not less than 20 methimazole tablets. Weigh accurately a portion of the powder equivalent to about 120 mg of methimazole and place in 100 ml volumetric flask. Add about 80 ml of water, insert the stopper and shake by mechanical means or occasionally by hand during 30 minutes, dilute with water to volume and mix. Filter and transfer 50.0 ml of the filtrate to a 125 ml conical flask. Add from a buret 3.5 ml of 0.1N NaOH, mix, and add with agitation about 7 ml of 0.1N AgNO₃. Add 1 ml of bromothymol blue T.S. and continue the titration with 0.1N NaOH until a permanent, blue green color is produced. Each ml of 0.1N NaOH is equivalent to 11.42 mg of C₄H₆N₂S.

ii) Iodometric methods

a) Kossakowski et al (20) developed the following method for determination of methimazole (thiamazole) in substances and tablets.

A solution containing 10-60 ug thiamazole was buffered at pH 5.6, treated with NaN₃ and then with 0.02 M iodine solution, left 10 minutes and titrated for unchanged iodine with 0.02 M Na₃AsO₃. The method was useful in determining methimazole in compound drugs provided they

contain no S^{-2} ions.

b) By the method Blazek et al (21)

The determination is done by oxidation of the -SH group with 0.1N iodine solution in $NaHCO_3$ solution visually with respect to strength or potentiometrically (the equivalence point is difficult to recognize) or in NaOH solution potentiometrically, which is more favorable. The error of the method for a weight of 10mg amounts is approximately 0.5%.

iii) Iodine complex method (22).

The potential colorimetric use of the iodine complexes of methimazole in $CHCl_3$ and CCl_4 is studied. The absorption maximum of methimazole-iodine complex at 269 nm could be used analytically.

iv) Bromometry method

The method was described by Varga et al (23). To a 2-5 mg sample in aqueous solution, 10 ml of water, 10 ml of 0.1N $KBrO_3$, and exactly 0.5 gm. KBr are acidified with 1.0 ml 50% H_2SO_4 . After 15 min. 5 ml 20% KI is added, with starch indicator and the iodine titrated with 0.1N $Na_2S_2O_3$. 1 ml 0.1 $KBrO_3$ = 0.0009517 methimazole. The bromometric determination of methimazole and other similar compounds is markedly influenced by the presence of excess bromide. In general, the greater the amount of bromide present the lower the percentage of the compound found. The deviation approaches a limit which is different for each compound (24).

v) Cerimetry method

The method was developed by Varga et al (23) 0.03 - 0.10 gm sample in 5 ml water is cooled to 0° and titrated with 0.1N $Ce(SO_4)_2$, using p-ethoxychrysoiodine or (not reversible) methyl orange, methyl red, or thymol blue as indicator. 1 ml 0.1N $Ce(SO_4)_2$ = 0.011417 gm methimazole. The error is less than $\pm 1\%$. Vehicles of tablets do not interfere.

6.12. Non-aqueous titration

A non-aqueous titration method was developed (25,26) for the determination of basic compounds with thio(-S-) and mercapto (-SH-) groups. The reaction of the S group with $\text{Hg}(\text{OAc})_2$ in acetic acid makes this possible. Methimazole and other compounds were all titrated with HClO_4 in acetic acid using gentian violet as indicator.

6.2 Spectrophotometric methods

6.21 Infrared spectrophotometric method

A method recommended for adoption as official, first action (27). The method in which methimazole is separated from tablet excipients by column chromatography on Celite 545 with chloroform as the eluent and then quantitatively measured and identified by IR spectrophotometry. This method was studied collaboratively by 10 analysts; average recoveries from two simulated and two tablets mixtures ranged from $96.6\% \pm 1.0$ to $101.1\% \pm 0.9$ (28).

6.22 Nuclear magnetic resonance spectrometric

An NMR procedure is described by which methimazole is determined in pure and tablet formulation. The method is rapid, accurate, precise (S.d. = 0.95%) and also provides a specific identification of methimazole.

The spectrum was run in 10% methylene chloride in carbon tetrachloride with use of benzoic acid as an internal standard, using the N-CH_3 protons of methimazole at 3.56 δ and the aromatic protons of benzoic acid at 7.5 and 8.13 δ as criteria for analysis (29).

6.23 Ultraviolet Spectrophotometric method

In this method, absorption spectra have been determined for methimazole (mercazoly) in aqueous acid and alkaline solutions. Aqueous solutions of methimazole have an absorption band in the medium wave region with a max at 250 nm. In strong acid and alkaline media hypsochromic and hypochromic effects are observed (30).

6.3 Chromatographic Analysis

6.31 Thin Layer Chromatography

Methimazole was determined in chloroform extracts of rat urine by thin layer chromatography on silica gel G. (containing 1% Zn Silicate phosphor) plates with $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (32:8:5) as developing solvent and 2,6-dichloroquinone chlorimide as detection reagent by densitometric scanning (31). This method is suitable for routine analysis (better than GLC because of the instability of the S. methylmethimazole derivative).

6.32 Gas Liquid Chromatography

This method is used for S-methyl methimazole on a 10% Apiezon L + 5% KOH/Chromosob W column at 100° with N carrier gas. The method is more sensitive and more precise than TLC method, but duplicate measurements must be made on the same day due to instability of S-methylmethimazole (32). Clarke (4) reported retention time of 0.43 relative to diphenhydramine under condition of methimazole described in the monograph.

6.33 Paper Chromatography

Clarke (4) described several solvents systems used for paper chromatography of methimazole as shown in Table 1.

Table 1.

Solvent System	Visualising Agent	R _f	Ref.
Acetate Buffer (pH 4.58)	ultraviolet iodoplatinate spray (white)	0.27	33
Phosphate Buffer (pH 7.4)	ultraviolet iodoplatinate spray (white)	0.00	34,35
Citric acid: H ₂ O: n-butanol (4.8g: 130 ml: 870 ml).	ultraviolet iodoplatinate	0.72	36,37

6.4 Colorimetric Analysis

In this method the reaction of diphenylpicrylhydrazine with methimazole (thiamazole) was sufficiently rapid for its use in a spectrophotometric assay. ⁻⁵ When the concentration of the reagent was 4×10^{-5} M and the molar ratio of methimazole to the reagent was 2 : 10, decoloration stopped after 90 minutes at 26°. The reaction mixture then contained bis (1-methylimidazol-2-yl) disulphide, the reagent, diphenylpicrylhydrazine. After several hours the decoloration started again giving rise to a multiplicity of colored compounds some of which were also formed in a methanolic solution of the reagent. Reaction mechanisms were proposed for equimolar amounts of the reactants as well as for the reaction of methimazole with excess of the reagent and for the reaction of the reagent with the disulphide of methimazole (38).

Szabo *et al* (39) described method for identification and determination of methimazole by conversion into its coloured 1:1 Cu^{+2} complex which has an absorption max. at 614 nm. The complex is not stable, but on treatment with HCl, a 2:1 methimazole CuCl complex is precipitated as white crystals.

6.5 Potentiometric Analysis

Methimazole was analysed in pharmaceutical preparations potentiometrically using 0.1N chloramine. The method can detect amounts of up to 5 mg of the drug with an accuracy of 98-99.5% (39).

An alternative method was described by Pinzauti *et al* (40) for determination of several antithyroid drugs potentiometrically with 0.01 M mercuric acetate with use of a mercuric sulfate reference electrode and an amalgamated gold or a silver indicator-electrode. The method is rapid and the results are reproducible; the errors are all within $\pm 0.36\%$.

6.6 Coulometric determination

Certain thiols, including methimazole, were determined coulometrically by direct titration with electro-generated Hg^{+2} (42). The coulometric assay is carried out in a cell having three compartments separated from each other by sintered-glass discs. One compartment contains the mercury for the electro-

generation of Hg^{+2} , the mercury indicator electrode and the S.C.E. (enclosed in a Perley tube containing 4 M-sodium nitrate to avoid interference with Cl^-); the second is filled with supporting electrolyte (an aqueous soln. 0.05 M in $\text{Na}_2\text{B}_4\text{O}_7$ and 0.5M KNO_3 and of pH 9.3); and the third acts as an auxiliary compartment. The counter-electrode is of polished platinum.

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NALIDIXIC ACID

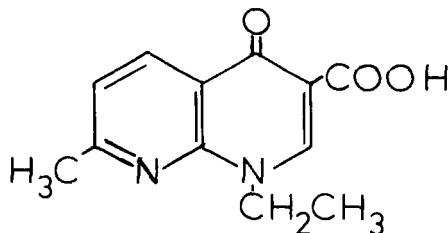
Patricia E. Grubb

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1. Description

1.1 Name, Formula, Molecular Weight

Nalidixic acid is 1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid.



Molecular Formula: $C_{12}H_{12}N_2O_3$

Molecular Weight: 232.24⁽¹⁾

1.2 Appearance, Color, Odor

Nalidixic acid is a white to slightly yellow, odorless crystalline powder.⁽¹⁾⁽²⁾

2. Physical Properties

2.1. Spectral Properties

2.11 Mass Spectrum

The low resolution mass spectrum of nalidixic acid is presented in Figure 1. It was obtained on a Joel JMS 01SC Mass Spectrometer at an ionization potential of 75 eV. The fragmentation pattern is presented in Figure 2. The molecular ion ($m/e=232$) is present at an intensity of 18%; the loss of CO₂ gives a fragment of mass 188 which is the most abundant ion.

2.12 Infrared

The infrared spectrum of nalidixic acid in a KBr pellet is presented in Figure 3. The spectrum was obtained on a Perkin-Elmer Infrared Spectrophotometer Model 21. It agrees with the spectrum presented by Salim and Shupe.⁽²⁾

MASS SPECTRUM
of Nalidixic Acid

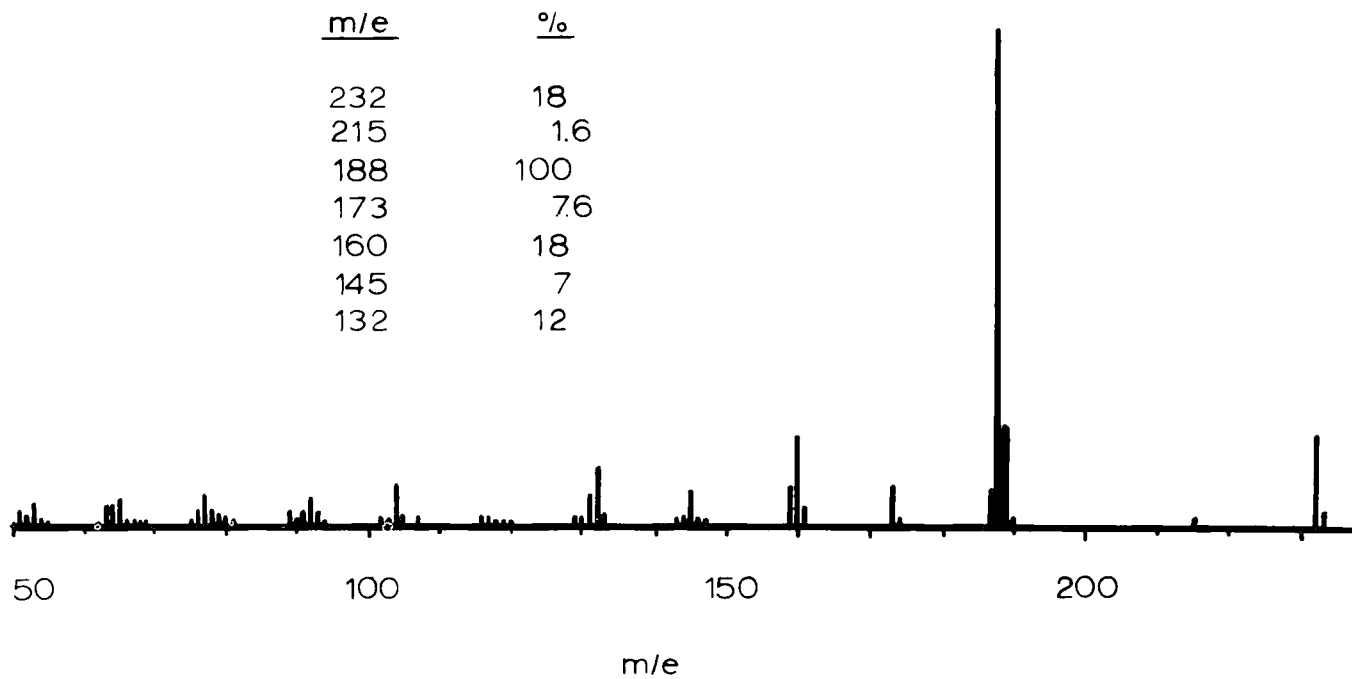


Figure 1

Figure 2

Fragmentation Pattern of Nalidixic Acid

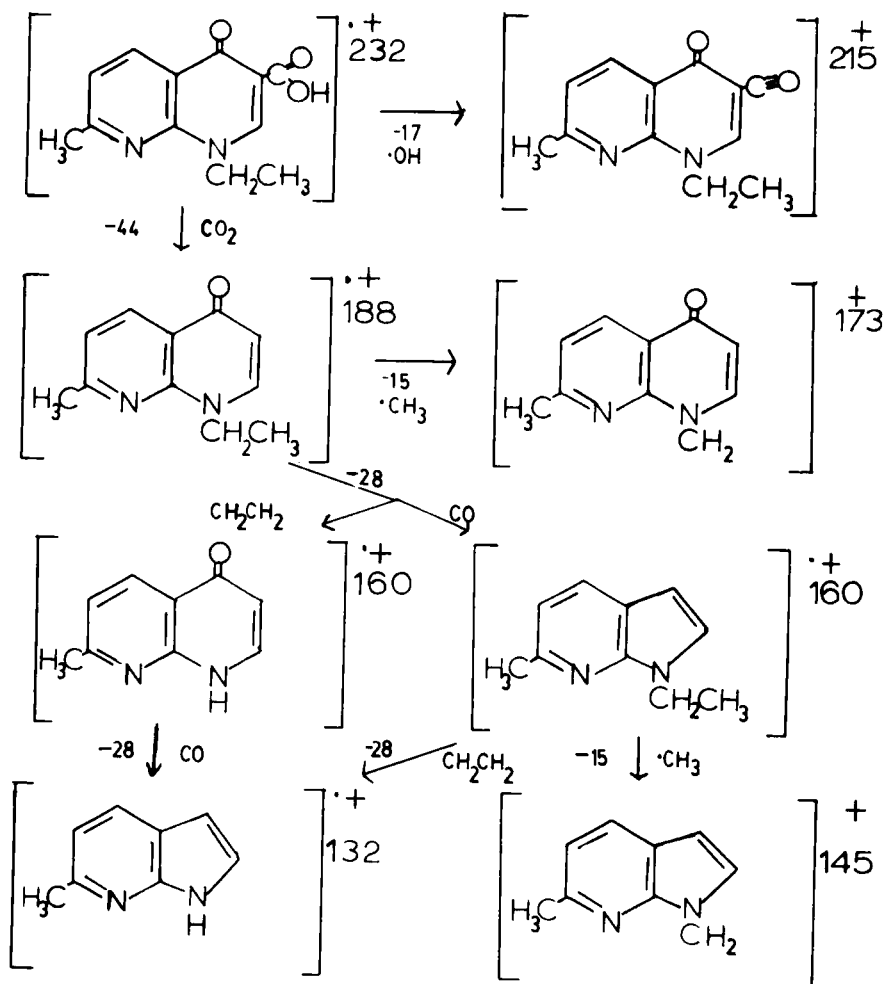
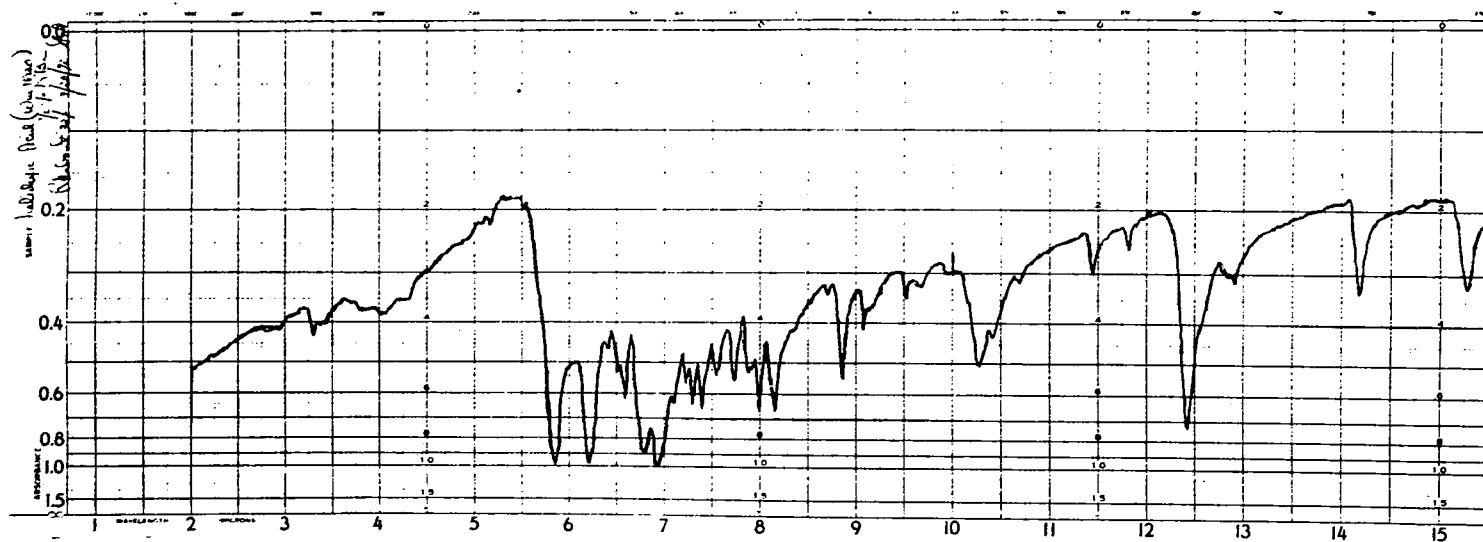


Figure 3

INFRARED SPECTRUM
of Nalidixic Acid



The carboxylic acid OH bands in the region of 3300-2500 cm^{-1} are weak and broad, indicating that hydrogen bonding with the carbonyl may be present.⁽³⁾ The intense peak at about 1715 cm^{-1} is probably due to the C=O stretching of the carboxylic acid. The peak at 1620 cm^{-1} may be due to the C=O stretch of the carbonyl at position 4 or the C=C stretch of C-2 and C-3, conjugated with the carbonyl, or a combination of these two vibrations.

2.13 NMR

The NMR spectrum of nalidixic acid is presented in Figure 4. It was obtained on a Varian A60 spectrometer. This spectrum is in agreement with the spectrum presented by Hamilton and co-workers.⁽¹⁴⁾ The following assignments have been made.

<u>ppm</u>	<u># protons</u>	<u>description</u>
1.75	3(triplet)	$-\text{CH}_2\text{CH}_3$
2.98	3(singlet)	$-\text{CH}_3$ (aromatic)
5.12	2(quartet)	$\text{N}-\text{CH}_2-\text{CH}_3$
7.85	1(doublet)	C(6)H
8.85	1(doublet)	C(5)H
9.47	1(singlet)	C(2)H

2.14 Ultraviolet Spectrum

Figure 5 shows the ultraviolet spectra of nalidixic acid at about 7.5 mcg/ml in 0.1 N NaOH, methanol, and chloroform, obtained on a Perkin-Elmer 323 recording spectrophotometer. The intensity, position, and fine structure present in each spectrum is related to the solvent polarity. These spectra are in agreement with the spectra published by Salim and Shupe.⁽²⁾ Zubenko and Shcherba also report that there are two bands in methanol and 0.1 N NaOH, at 258 nm and 324 or 332 nm, respectively.⁽⁵⁾ Gafari⁽⁶⁾ has reported three bands in methanol: 213-216 nm (^1La), 255 nm (^1Lb) and 320-322 nm (^3Lc). Gafari also reports an absorbance in 0.1 N NaOH at 279-281 nm and 325 nm. These wavelengths are about 3-10 nm lower than those reported by other sources.⁽²⁾⁽⁵⁾⁽⁷⁾

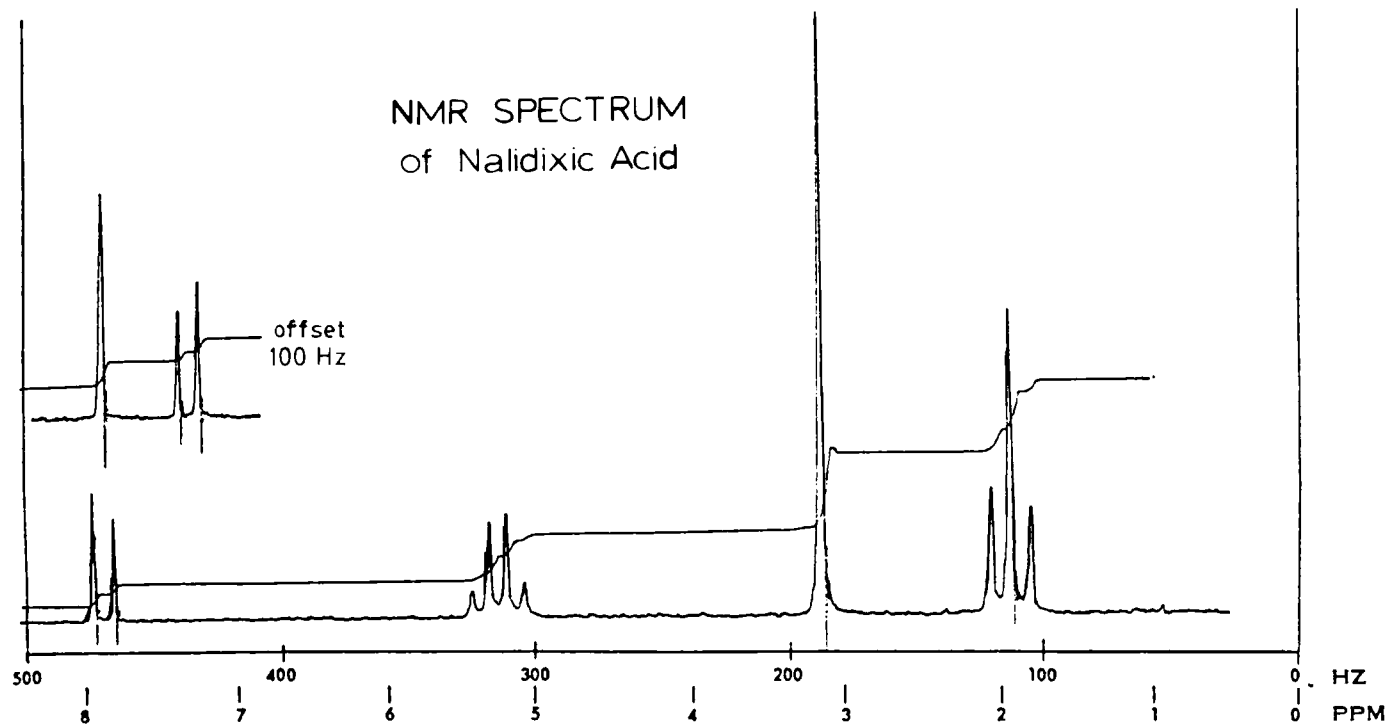


Figure 4

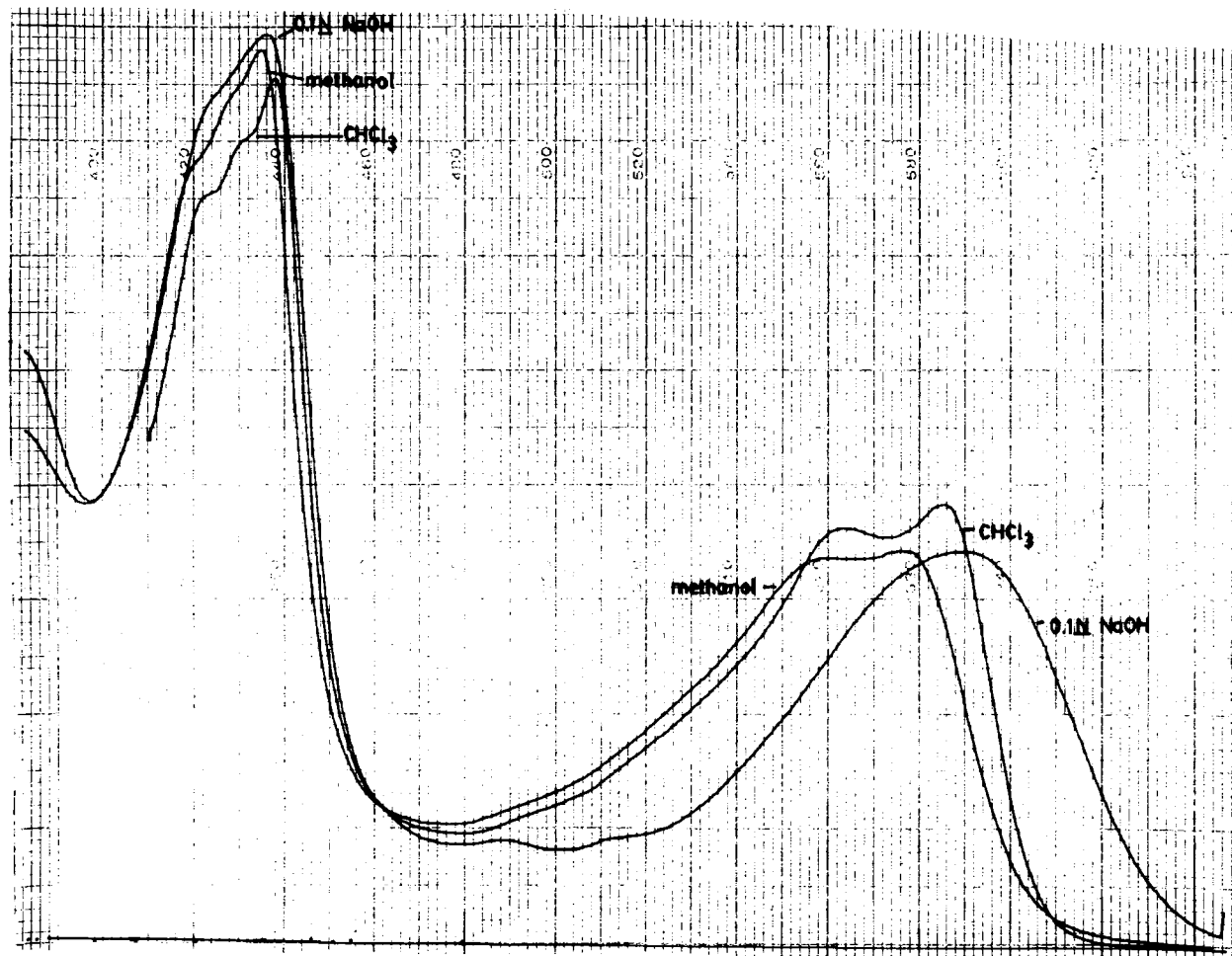


Figure 5.
ULTRAVIOLET SPECTRA
OF NALIDIXIC ACID

2.15 Fluorescence

Nalidixic acid exhibits strong fluorescence in acidic solutions. McChesney and co-workers used 0.1 N H_2SO_4 (8) with an excitation wavelength of 330 nm, measuring emission at 375 nm. Browning (9) used 21.5 N H_2SO_4 with excitation at 325 nm and emission at 408 nm. Figure 6 shows the fluorescence excitation and emission spectra of nalidixic acid in 0.5 N H_2SO_4 determined on an Aminco Bowman Spectrophotofluorimeter.

2.2 Crystal Properties

2.21 Crystallinity and X-ray Diffraction

Prismatic crystals of nalidixic acid elongated along the c axis were grown in ethanol/water solution by Achari and Neidle. (10) The crystals were found to be monoclinic and of the space group $P2_1/C$. The x-ray diffraction pattern of the single crystal was solved for the structure of the molecule. The bond lengths and angles of all non-hydrogen atoms were determined. The values found compare favorably with those reported for 1,8-naphthyridine and 3-ethoxycarbonyl-4-oxo-6-methyl homopyrimidazole. Nalidixic acid is calculated to be slightly non-planar; each ring is planar but the ring fusion has induced a slight buckling of the ten-membered ring. The above-mentioned compounds also have similar buckling. An intramolecular hydrogen bond is found between the carbonyl oxygen and the hydrogen of the carboxylic acid.

The parameters of the monoclinic crystal are

$$a = 8.913 \text{ \AA}$$

$$b = 13.133 \text{ \AA}$$

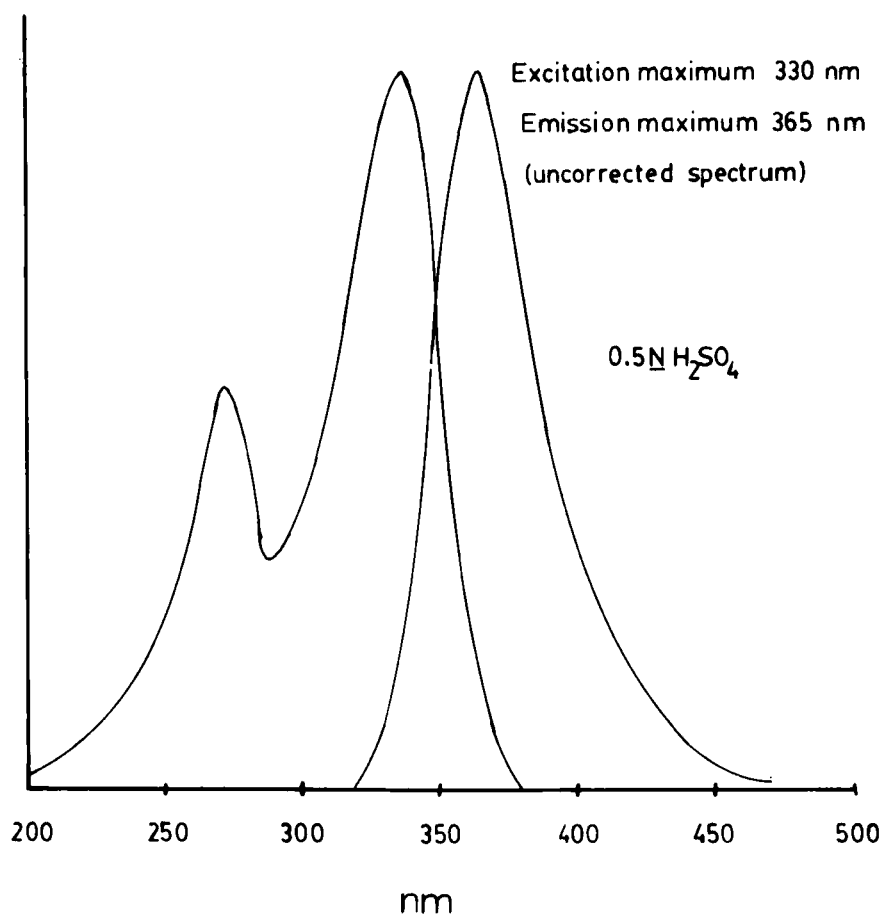
$$c = 9.31 \text{ \AA}$$

$$\beta = 99.75^\circ$$

The measured density is 1.41, the calculated density is 1.425 g/cm^3 for $Z=4$. The refractive indices of crystals of nalidixic acid have been reported as $\alpha=1.510$, $\beta=1.800$, and $\gamma=1.880$. (4) (11)

Figure 6

FLUORESCENCE SPECTRUM of Nalidixic Acid



2.22 Solubility

The solubilities of nalidixic acid in various solvents at 23° are listed below.

<u>Solvent</u>	<u>Solubility (mg/ml)</u>
chloroform	35
toluene	1.6
ethyl acetate	.8
methanol	1.3
ethanol	.9
isopropanol	.4
water (distilled)	.1
ethyl ether	.1

The partition coefficients between water and various organic solvents have been reported by Sulkowska and Staroscik.(12)

The pH-solubility profile has been studied by these workers and also by Takasugi and co-workers.(13)

2.23 pKa

The pKa of the protonation of the nitrogen in position 8 has been reported as 6.02 and the pKa for the carboxylate anion formulation has been reported as -0.94. These were determined by Staroscik and Sulkowska by a spectrophotometric method.(14) Further study by the same workers on the partition equilibria of nalidixic acid between water and various organic solvents led to calculations of the pKa values of 5.99 ± 0.03 for N-protonation and -0.86 ± 0.07 for carboxylate anion formation.(12) Takasugi and co-workers reported the apparent pKa of nalidixic acid to be 5.9 at 28° by a spectrophotometric method.(13)

2.24 Melting Range

The melting range of nalidixic acid is reported to be 225-231°, determined as a class 1 compound.(1)(2)

2.25 Differential Scanning Calorimetry

A DSC scan for nalidixic acid was performed by Houghtaling.⁽⁹⁾ The instrument used was a Perkin-Elmer DSC-1B at a scan rate of 1°/min. The curve is presented in Figure 7. A sharp peak at 229-230.5° (corr) represents the sample melting.

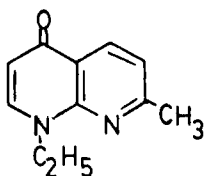
3. Synthesis

The synthesis of nalidixic acid was reported by Leshner and Gruett.⁽¹⁵⁾⁽¹⁶⁾ It may be prepared by the procedure shown in Figure 8.

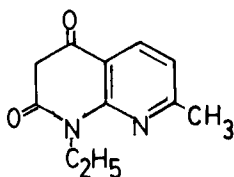
4. Stability and Degradation

Nalidixic acid is stable up to five years under reasonable conditions of temperature and humidity. Pawelczyk and Plotkowiakowa⁽¹⁷⁾ subjected sodium nalidixate solutions to accelerated aging, but were unable to identify decomposition products. Detzer and Huber⁽¹⁸⁾ studied the photolysis and thermolysis of nalidixic acid in the presence of oxygen. Photolysis produced de-carboxylated nalidixic acid, structure A, and a diketone product, structure B, as well as carbon dioxide and ethylamine.

Structure A



Structure B



Thermolysis also produced the decarbonylation product plus a dimer, structure C.

Structure C

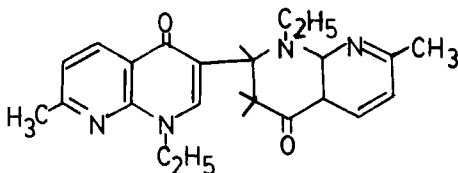


Figure 7

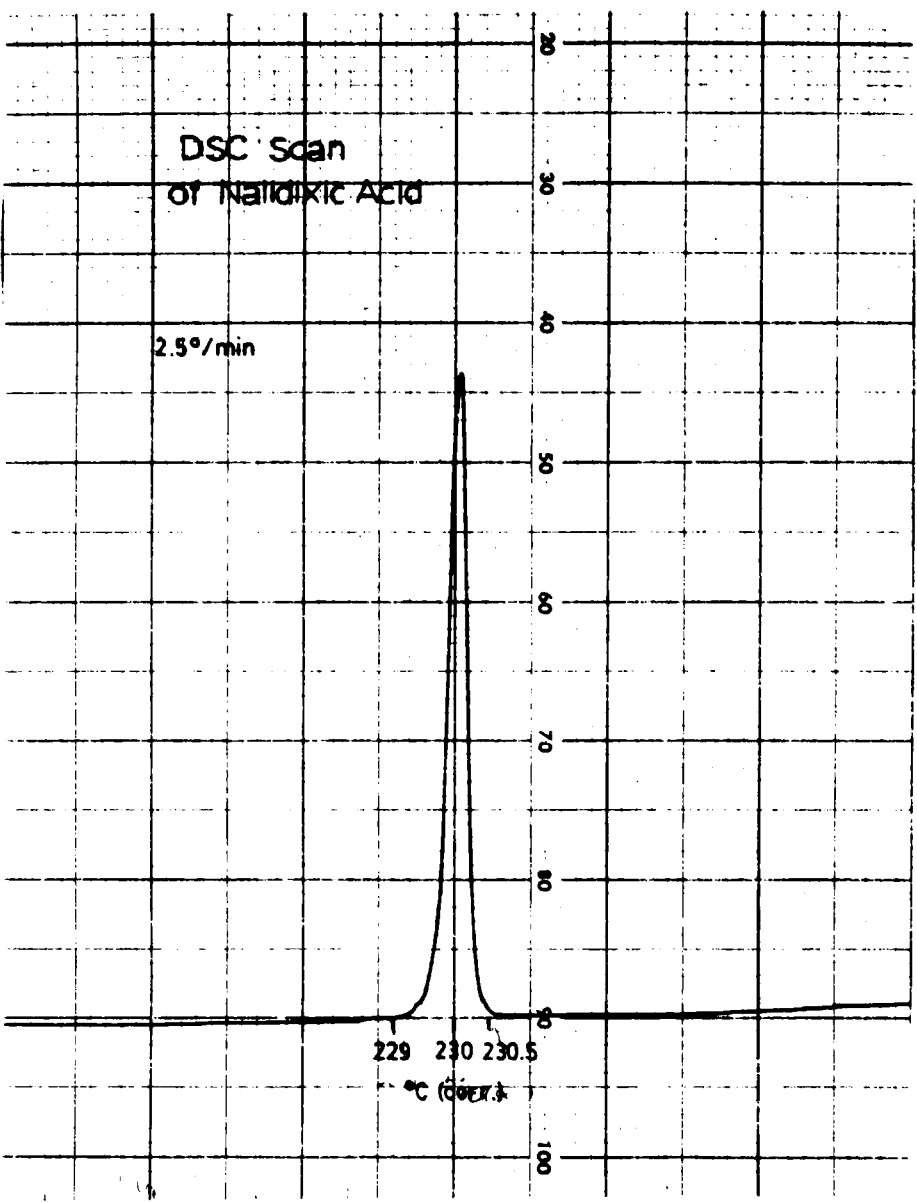
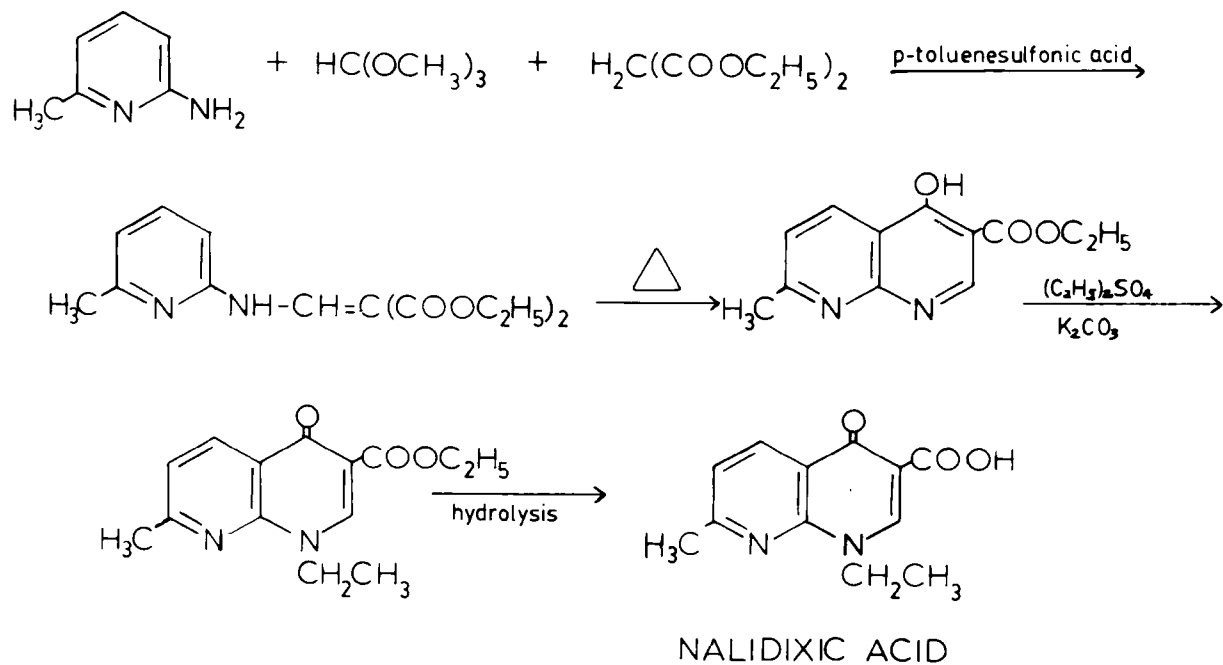


Figure 8
SYNTHESIS of NALIDIXIC ACID



5. Drug Metabolic Products and Pharmacokinetics

5.1 Metabolic Products

Nalidixic acid is a synthetic antibacterial compound that is used in the treatment of urinary tract infections. It is more active against gram-negative than gram-positive organisms.⁽⁴⁾ The compound is rapidly absorbed as the free acid; it is excreted in the urine by man in several forms.⁽⁸⁾ A small amount is excreted unchanged, but a much larger fraction is conjugated as the monoglucuronide. The most important metabolite is 1-ethyl-1,4-dihydro-7(hydroxymethyl)-4-oxo-1,8-naphthyridine-3-carboxylic acid, referred to as hydroxynalidixic acid. This metabolite has shown the same order of microbiological activity in vitro as nalidixic acid. The monoglucuronide conjugate of 7-hydroxynalidixic acid has also been found, as well as the 3,7-dicarboxylic acid product. No antibacterial activity has been found for the glucuronides or for the 3,7-dicarboxylic acid. In addition, no glucuronide of the 3,7-dicarboxylic acid has been reported.

Similar metabolites were found produced by man⁽¹⁹⁾⁽²⁰⁾⁽²¹⁾, monkeys⁽⁸⁾, dogs⁽⁸⁾, chickens⁽²²⁾, calves⁽²³⁾ and microorganisms⁽⁴⁾. The ratios of these metabolites, however, were found to vary with the individual. The overall conversion of nalidixic acid to hydroxynalidixic acid had been reported by McChesney⁽⁸⁾ to be normally about 32%; bicarbonate supplementation increased this to about 40%. Bicarbonate supplementation also increased the amount of total naphthyridine excreted in the biologically active form.

Two separate studies have reported on the ratios of the various metabolic products excreted in the urine by man. In addition, Portmann and co-workers calculated theoretical amounts based on their rate equations.⁽²⁴⁾

Compound	Calc. amts. (mg per lg dose)	Found(24) (mg per lg dose)	Found(21) %
Nalidixic acid	9	8 \pm 3	0.5-5 %
Hydroxy-nalidixic acid	105	129 \pm 8	2.5-6 %
NA-glucuronide	517	537 \pm 49	24-80%
1-HNA-glucuronide	221	229 \pm 32	11-12%
3,7-dicarboxylic acid	74	43 \pm 6	not reported

In glucuronides were not found as metabolic products by Hamilton⁽⁴⁾ in studies of various fungi. The major metabolite of the microorganisms was hydroxy-nalidixic acid. The dicarboxylic acid was also found as a product in a number of microorganisms.

The mechanism of action of nalidixic acid against *E. coli*. was found to be inhibition of DNA synthesis.⁽²⁵⁾ No selective effect on purines or pyrimidines was found and no inhibition of initiator synthesis was demonstrated. In addition the nalidixic acid could be removed from cultures after exposures up to 75 minutes by rinsings and cells would recover from the block.

5.2 Pharmacokinetics

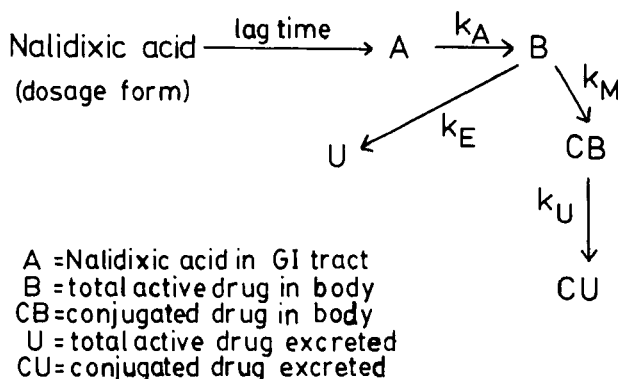
The pharmacokinetics of nalidixic and hydroxy-nalidixic acids have been studied by several different groups. Takasugi *et al* studied *in-situ* and *in-vitro* absorption of nalidixic acid from the gastrointestinal tracts of rats as a function of pH. They reported that the absorption of non-ionized nalidixic acid was faster than the ionized form, with the maximum absorption rate constant found when the drug was administered from a pH=3 buffer solution. The absorption *in-situ* was found to be ten times the rate *in-vitro*, but this was dependent on several factors.⁽¹³⁾

Moore and co-workers⁽¹⁹⁾ described a simplified model for nalidixic acid metabolism by combining the

two microbiologically active forms, nalidixic and hydroxynalidixic acid, and the two conjugated products of the active forms. This model is shown below in Figure 9.

Figure 9

Simplified Model for Kinetic pathways of
Nalidixic acid in Man



The rate constants for metabolism and excretion were further combined to a rate constant for the disappearance of the drug, k_d . Four dosage forms were studied and it was found that k_d was constant for all forms except for a coarse, slowly dissolving powder.

Portmann and co-workers then studied the kinetic pathways in man for hydroxynalidixic acid, the active primary metabolite.⁽²⁶⁾ The rate constants for glucuronide formation, oxidation to the dicarboxylic acid and excretion of hydroxynalidixic acid were calculated. Essentially total absorption of hydroxynalidixic acid was found in every case. Good agreement between experimental and theoretical plasma levels, based on the first order rate approximations used for the model, was found. Again, the disappearance rate constant, k_{d2} , was found to be very similar for each subject, although the individual excretion and metabolic rate constants varied widely. The disappearance rate constant, k_{d2} , was defined as the sum of the excretion rate constant, k_{E2} , and the metabolic rate constants to the glucuronide and dicarboxylic acid, k_{M3} and k_{M4} , respectively.

This data was then used in another study by Portmann and co-workers(24) of nalidixic acid metabolism in man in which a more elaborate model was developed and various rate constants were reported (Figure 10). This model was based on the oral administration of 1 g of nalidixic acid. Theoretical curves for plasma levels of nalidixic and hydroxynalidixic acid vs. time agreed with experimental values.

McChesney and co-workers(27) then studied the effect of repeated oral dosage of nalidixic acid and found some carry-over of nalidixic acid from day to day but reported no important change in the metabolism due to multiple dosings.

The half-life of nalidixic acid in plasma in man was found to be between 85 and 100 minutes by McChesney and co-workers(8) and was reported as about 100 minutes by Bruehl and co-workers(27).

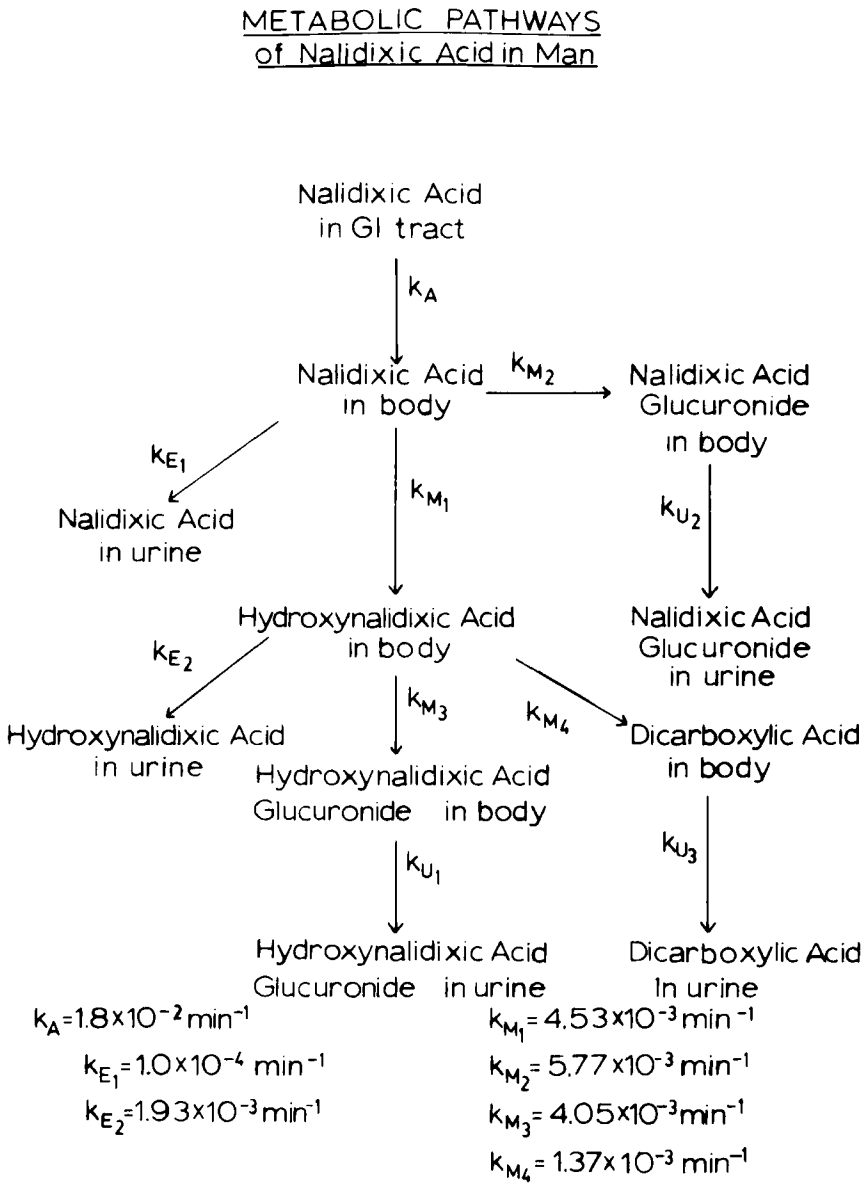
Another study by McChesney and co-workers on the metabolism of nalidixic acid in the immature calf(23) demonstrated a pattern of metabolism very different than in man. The half-life of nalidixic acid was found to be about 24 hours and a large amount was excreted into the feces. The immature calves were also unable to excrete nalidixic acid into the urine at concentrations greater than found in the plasma and conjugated drug was present at low levels only. Calves seven months old had metabolic patterns much closer to man; the plasma half-life was about 1.5 hours, the concentration of excretion into the urine was at least ten times that in plasma and the extent of conjugation was increased. The inability to metabolize nalidixic acid by the immature calf was considered to be due to incomplete development of its metabolic system. A similar effect was seen in human infants.(29)

6. Methods of Analysis

6.1 Elemental Analysis

The molecular formula is $C_{12}H_{12}N_2O_3$.

Figure 10



<u>Element</u>	<u>Theory</u>	<u>Found for standards</u> ⁽³⁰⁾	
C	62.07%	62.11	61.98
H	5.21%	5.21	5.20
N	12.07%	12.00	12.32

6.2 Non-aqueous Titration

The titration of nalidixic acid in DMF with lithium methoxide has been reported⁽¹⁾⁽²⁾ with thymolphthalein as the indicator. It has also been titrated with sodium methoxide in ethylenediamine or DMF:methanol 1:2 with thymol blue indicator.⁽³¹⁾ An error for this titration was reported as + 0.7%. A titration with sodium borohydride followed potentiometrically or with thymol blue indicator has also been reported by Bachrata and co-workers. The standard deviation was reported as + 0.60%.⁽³²⁾

6.3 Spectrophotometric

The ultraviolet absorption spectrum of nalidixic acid in methanol or chloroform has an absorption maximum at about 258 nm and a broad double peak at 324 to 333 nm. In 0.1 N NaOH the band at 324 nm is shifted to a single peak at about 332 nm. The a of the band at about 258 nm is approximately 110 but varies with the solvent.⁽²⁾⁽⁵⁾⁽⁶⁾⁽⁷⁾

6.4 Colorimetric

Nalidixic acid and sodium nalidixate form a strong colored complex with iron III. The maximum of absorbance of the complex is 410 nm and Beer's Law is obeyed from 10 to 250 μ g of nalidixic acid per ml⁽³³⁾, and from 0.43 to 17.05 mg iron III per ml.⁽³⁴⁾ Nalidixic acid complexes through the oxo-group on C-4 and the carboxylic acid group on C-3. Three moles of nalidixic acid complex with one mole of iron III. The instability constant of the complex was calculated to be 2.11×10^{-8} by Dick and Murgu.⁽³⁴⁾

6.5 Polarographic

The polarographic behavior of nalidixic acid has been studied by Staroscik and co-workers.(35) The pH range of -2.9 to 11 in 20% DMF was investigated in the concentration range of $5 \times 10^{-4}M$ and three stages of reduction were found. The potentials were found to vary linearly with pH for the first two reduction stages, while the third was constant and appeared at pH >8. The carbonyl on C-4 was shown to be reduced to the 4-hydroxy product. Nalidixic acid was reduced with sodium borohydride and the product was demonstrated to be the same as that in the polarographic reduction by TLC.

6.6 Chromatographic

6.61 Thin-layer and paper chromatography

Several thin-layer and paper chromatographic systems for nalidixic acid are listed below.

Chromatographic Systems for Nalidixic Acid

<u>Adsorbent</u>	<u>Mobile Phase</u>	<u>R_f</u>	<u>Ref.</u>
paper	toluene:dioxane(9:1) descending	.6	8
paper	butanol:acetic acid: H ₂ O (4:1:1)	--	36
paper	propanol:ethanol:water (6:1:3)	--	36
paper impreg. with 0.1 M Na and HPO ₄	iso-butanol:ethanol:ethyl acetate:water:acetone (8.2:1:5:2) ascending	.73	17
silica gel	methanol:water:ammonia (100:12:16)	.88	35
silica gel	chloroform:methanol:formic acid (90:7:3)	.54	37
silica gel	ethyl acetate:methanol: isopropylamine (76:20:4)	.18	37
silica gel	benzene in ethanol:acetic acid (80:10:10)	.5	23

6.62 Liquid chromatography

A high-performance liquid chromatographic method for nalidixic acid on a strong anion-exchange resin column has been reported, using a mobile phase of 0.01 M sodium tetraborate at pH 9.2 and 0.003 M sodium sulfate. The relative retention time for nalidixic acid in the system reported by Sondach and Koch was 0.86 with sulfanilic acid as the standard at 1.00.(38)

6.63 Gas chromatography

A gas chromatographic procedure for nalidixic acid after esterification has been reported for biological samples.(39)

6.7 Spectrofluorimetric

Nalidixic acid has a strong fluorescence spectrum which has been used for its determination in biological fluids.(8)(9)(24)(26)(40)

7. Identification and Determination in Biological Fluids

The first spectrofluorimetric methods reported for the determination of nalidixic acid and its metabolites in biological fluids did not differentiate between nalidixic acid and hydroxynalidixic acid. The determination of free nalidixic acid and the hydroxy-metabolite in human urine plasma and feces was performed by extraction by toluene from acidified biological fluid and subsequent fluorimetric measurement at 325/375 nm of sample re-extracted into aqueous solution.(8) Conjugated nalidixic and hydroxynalidixic acids were determined by acid hydrolysis and then toluene extraction for fluorimetric measurement of the total drug. The conjugated nalidixic acid was then determined by difference.

A refined method involving extraction of two aliquots of biological material, buffered at two different pH's, by toluene, and re-extraction into aqueous solution allowed the simultaneous determination of nalidixic and hydroxynalidixic acid(24)(26) by their differential extractabilities and fluorescent intensities. This method was extended to the differential determination of the conjugated forms of nalidixic and hydroxynalidixic acid.

The 3,7-dicarboxylic acid metabolite was also measured fluorimetrically by extraction with ethyl acetate:chloroform 1:1 at pH=1 and re-extraction into aqueous solution. The fluorescence was determined at 350/435 nm; there was no cross interference with the nalidixic acid determination.(8)

Extraction of nalidixic acid with chloroform from urine has also been reported.(40) Another fluorimetric method for chicken liver and muscle containing not less than 100 ppb nalidixic acid was reported by Browning(9) using an ethyl-acetate extraction and alumina column to retain the nalidixic acid. The fluorescence was measured at 325/408 nm.

Spectrophotometric measurements of nalidixic acid have been reported. Gafari et al(41) used an extraction with ultraviolet measurement at 255 or 327 nm for blood and tissue samples. Takasugi and co-workers extracted buffered tissue homogenate with chloroform and measured the optical density at 334 nm.(13)

Several chromatographic procedures have been used for nalidixic acid and metabolites in biological fluids. Urine samples were extracted with chloroform and chromatographed on filter paper using the system toluene dioxane (9:1) descending chromatography. The known spots were eluted off the paper with methanol and quantitatively measured spectrophotometrically.(8) Other chromatographic systems reported include butanol:acetic acid:water (4:1:1) and propanol:ethanol:water (6:1:3) for paper chromatography. The glucuronides were also separated without prior hydrolysis using isopropanol:ethyl acetate:water (6:1:3) or propanol:ethyl acetate:acetic acid:water (5:5:1:3).(36)

A high performance liquid chromatographic method was developed by Shargel and co-workers for the assay of nalidixic and hydroxynalidixic acids in human plasma and urine.(42) Extraction into chloroform and re-extraction into aqueous solution was used as the sample treatment. The column used was a 0.5 M stainless steel (2.1 mm i.d.) packed with Zipax SAX strong anion exchange resin. The column pressure was 600 psi with a flow rate of 0.8 ml/min. A modification of this procedure, using a 1 m column with a column pressure of 900 psi and a flow rate of 0.7 ml/min. and an internal standard of 1-ethyl-1,4-dihydro-4-oxo-7-(4-pyridyl)-3-quinolinecarboxylic acid, was used by Goehl and co-workers(43) for improved separation. A separate liquid chromatographic procedure for the dicarboxylic acid was used by Lee(44) et al. The column used was a 25 cm x 4.6 mm i.d.

Partisil PXS 10/25 PAC pre-packed Magnum 9 column with a mobile phase of methanol:pH 3 0.1 M citrate buffer 85:15 with a flow rate of 1.6 ml/min. The retention time of the dicarboxylic acid was 12 minutes.

A thin-layer gas chromatographic system was devised by Pittman and Shekosky⁽³⁹⁾ for chicken tissue and feces. A TLC system of benzene:methanol:acetic acid (9:1:1) was used for prior separation; the spots were then removed and esterified in 14% BF₃ in methanol. A 4 ft. 3% OV-17 column at 240° was used. Retention times of about 7 minutes for nalidixic acid, 10 minutes for hydroxynalidixic acid and 17 minutes for the dicarboxylic acid were reported.

A pulse polarographic system for detection of the dicarboxylic acid in the presence of nalidixic and hydroxynalidixic acids was devised by Koss and Warner.⁽⁴⁵⁾ The reduction potential in the system used was -0.54V vs. SCE.

Microbiological assay procedures for nalidixic acid have also been used for biological samples. Since nalidixic and hydroxynalidixic acids have the same order of antibacterial activity in-vitro, then cannot be determined separately.

<u>Organism</u>	<u>Reference</u>
P. bovisseptica	8
B. pumilus	46
E. coli	25,47, 48,49

8. Identification and Determination in Dosage Forms

Nalidixic acid has been determined spectrophotometrically in tablets after chloroform extraction at a wavelength maximum of 258 nm.⁽¹⁾⁽²⁾ Another source reported 259 nm as the maximum for chloroform and 258 nm for 0.1 N NaOH.⁽⁷⁾ The infrared spectrum has also been used to identify nalidixic acid in tablets.⁽⁷⁾

Non-aqueous titration of tablet extracts with sodium methoxide⁽²⁷⁾ or lithium methoxide⁽¹⁾⁽²⁾ has been reported. A polarographic determination of nalidixic acid in tablets at -0.6V vs. SCE in 20% aq. DMF with 0.1 N HCl has also been used.⁽³⁵⁾

High performance liquid chromatography was used by Sondack and Koch⁽³⁸⁾ to assay nalidixic acid in aluminum hydroxide gel suspension.

A microbiological detection has been described for use for pharmaceutical preparations of nalidixic acid. A diffusimetric determination using *E. coli* (Bruxelles) was reported by Monciu.⁽⁴⁸⁾

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NEOMYCIN

William F. Heyes

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1. Description.

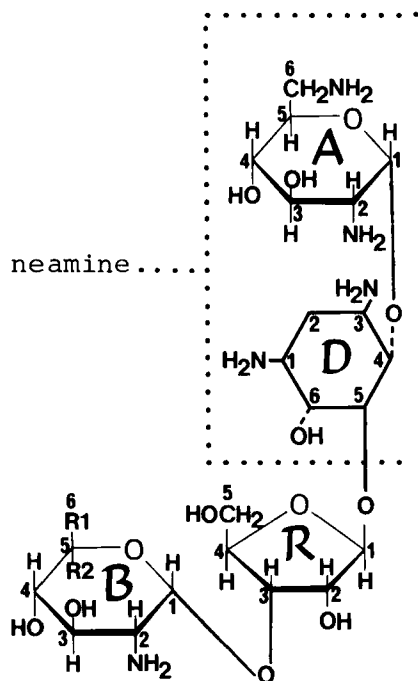
1.1. Composition, Name, Formula, Molecular Weight

Composition

Commercial neomycin is a complex mixture of aminoglycoside antibiotics originally isolated from a culture of *Streptomyces fradiae* by Waksman and his co-workers in 1949. The principle components of the mixture are neomycin B(I) and neomycin C(II) together with a small quantity of neamine¹, a degradation product of neomycin formerly known as neomycin A. Table 1 shows the content variability of neomycin B and C and neamine in commercial samples of neomycin as reported in the literature. Neomycins LP-A, LP-B and LP-C which chemically are the mono N-acetyl derivatives of neomycins A, B and C² may also be present in small amounts. Several other minor components have recently been identified as paromamine, paromomycin I and paromomycin II³. (Also known as neomycins D, E and F respectively).

Table 1
Content Variability of Neomycin

<u>Source</u>	<u>Neomycin B Content %</u>	<u>Neomycin C Content %</u>	<u>Neamine Content %</u>	<u>Reference</u>
Canada	69.2 to 97.5	30.8 to 4.0	Nil	5
U.K.	80.5 to 92.0	17.5 to 8.0	0 to 2.5	6
U.S.S.R.	63.7 to 91.6	40.0 to 3.4	Nil	7,8



- I $R_1 = H$ $R_2 = CH_2NH_2$; neomycin B
 II $R_1 = CH_2NH_2$ $R_2 = H$; neomycin C

Figure 1. Structure of neomycin and neamine

Names

The following are alternative names for the neomycin complex, all of which have been used by Chemical Abstracts:-

Colimycin, Colistin(not to be confused with Polymixin), Dextromycin, Flavomycin, Fradiomycin, Framycetin, Mycerin, Mycifradin, Neomix, Soframycin, Streptothricin BI, BII.

Chemical Names⁴

Neomycin B	O-2,6-diamino-2,6-dideoxy- α -D-glucopyranosyl-(1 \rightarrow 4)-O-[O-2,6-diamino-2,6-dideoxy- β -L-idopyranosyl-(1 \rightarrow 3)- β -D-ribofuranosyl-(1 \rightarrow 5)]-2-deoxy-D-streptamine.
Neomycin C	O-2,6-diamino-2,6-dideoxy- α -D-glucopyranosyl-(1 \rightarrow 4)-O-[O-2,6-diamino-2,6-dideoxy- α -D-glucopyranosyl-(1 \rightarrow 3)- β -D-ribofuranosyl-(1 \rightarrow 5)]-2-deoxy-D-streptamine.
Neamine (formerly Neomycin A)	2-deoxy-4-O-(2,6-diamino-2,6-dideoxy- α -D-glucopyranosyl)-D-streptamine.

Chemical Abstracts Registry No.

Neomycin B	119-04-0
Neomycin C	66-86-4
Neamine	3947-65-7

Formula

Neomycin B,C	C ₂₃ H ₄₆ N ₆ O ₁₃
Neamine	C ₁₂ H ₂₆ N ₄ O ₆

Molecular Weight

Neomycin B,C	614.67
Neamine	322.36

1.2. Appearance, Colour, Odour, Taste

The sulphate salt of neomycin (the usual commercial form) is an amorphous, white odourless, powder which is practically tasteless.

1.3. Definition of International Standard

The International Reference Standard for neomycin⁹ is defined as containing 680 units of activity per mg or alternatively 1 unit of activity is contained in 0.00147mg of standard material.

Similarly, the International Reference Standard for neomycin B is defined as containing 670 units per mg, or alternatively 1 unit of activity is contained in 0.001492 mg of standard material.

2. Physical Properties

2.1. Spectra

2.1.1. Infra-Red Spectrum

The solid state infrared spectra of neomycins B & C sulphate have been recorded as a dispersion in potassium bromide and are illustrated in Fig. 2 and 3.

The infrared spectrum of neamine sulphate (formerly called neomycin A) also as a potassium bromide dispersion is illustrated in Fig. 4. All spectra are of authentic material supplied by The Upjohn Company, Kalamazoo.

Sammul et al¹⁰ have published the infrared spectrum of neomycin undecylenate.

2.1.2. Ultraviolet Spectrum

No absorption maxima in the ultraviolet region 200-340nm is observed with solutions of neomycin.

2.1.3. NMR Spectrum

The proton NMR spectrum of neomycins B and C has been used by Rinehart and co-workers¹ to elucidate the structure and

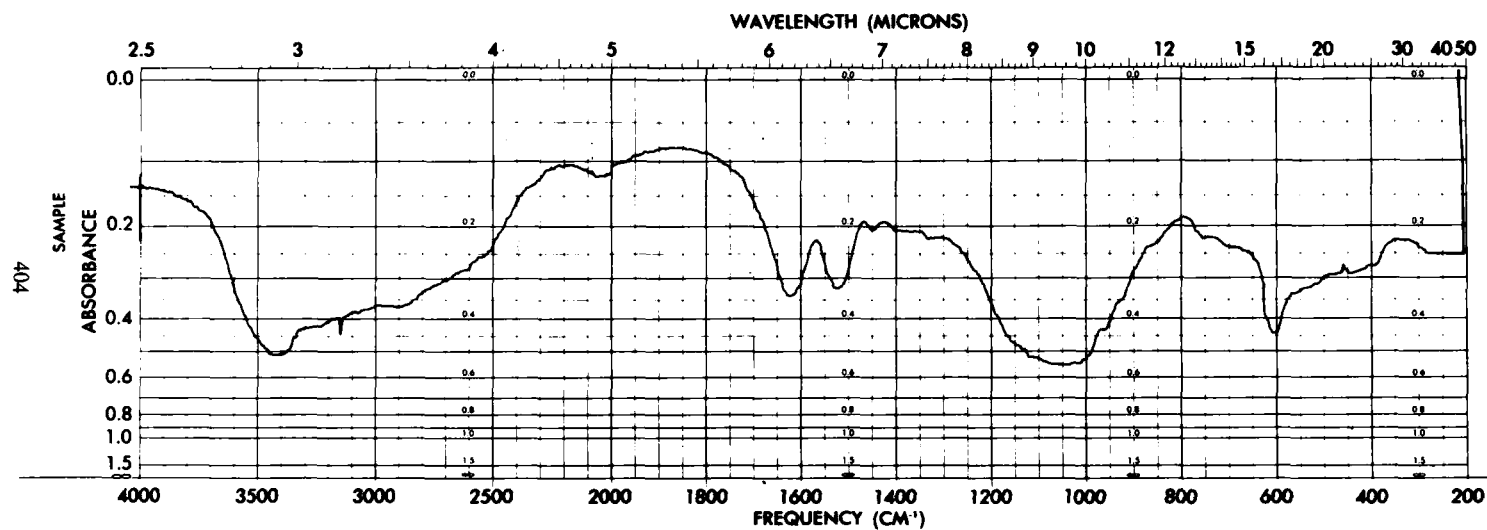


Figure 2. Infrared Spectrum of Neomycin B

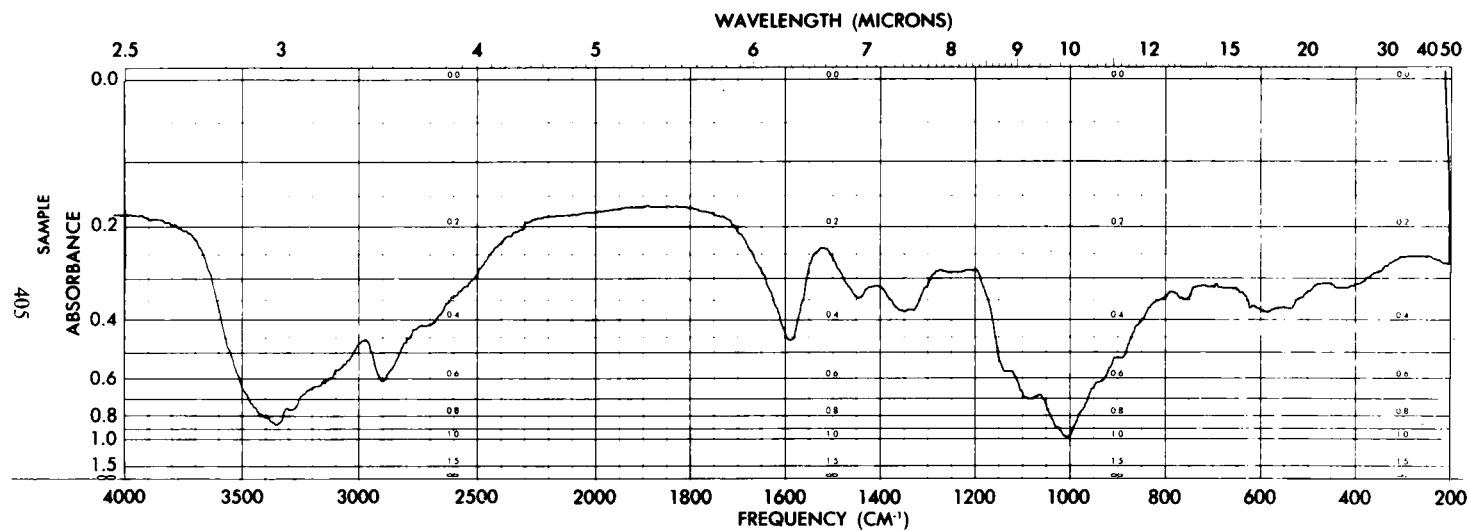


Figure 3. Infrared Spectrum of Neomycin C

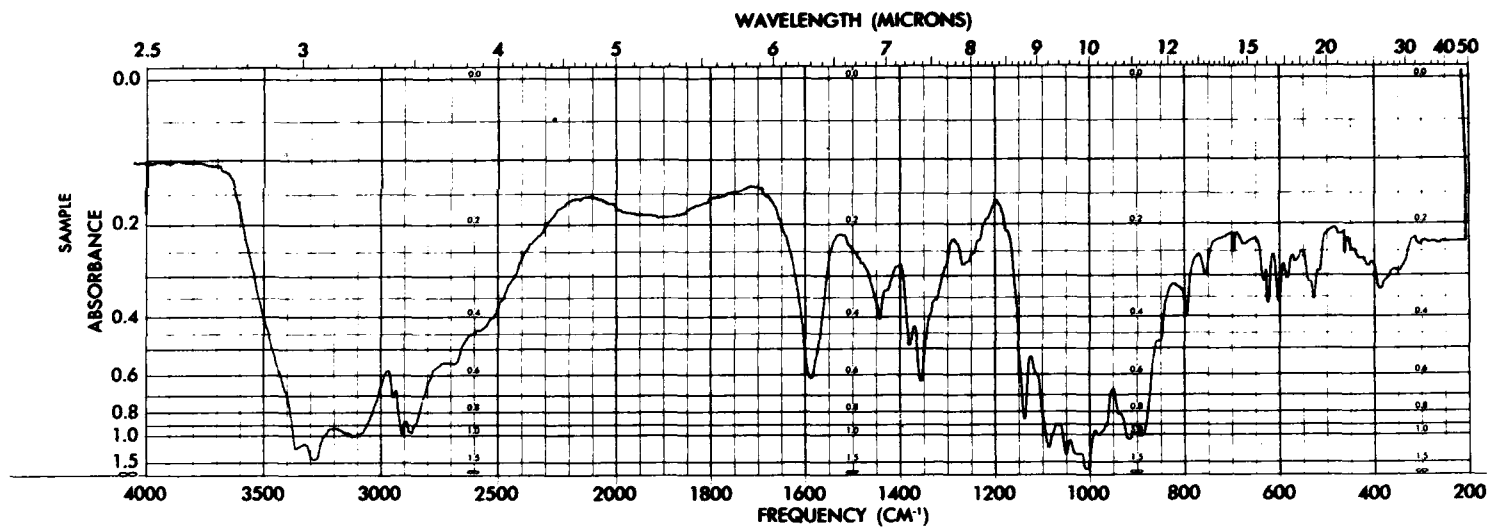


Figure 4. Infrared Spectrum of Neamine

stereochemical configuration of these antibiotics.

Truitt¹² has extensively studied the ¹³C NMR of the neomycins and demonstrated the pH dependence of the spectrum of neomycins B and C. The spectra of the hexa N-acetyl derivatives however, were shown to be independent of pH thus allowing assignment of the observed chemical shifts as given in Table 2.

2.14. Mass Spectrum

Neomycin is insufficiently volatile for direct mass spectrometric analysis. To overcome this problem Inouye¹⁴ prepared the volatile N-salicylidene Schiff's base, the M.S. of which, however, did not exhibit a peak for the molecular ion. To observe the molecular ion it was necessary to use the o-trimethylsilyl ether of the N-salicylidene Schiff's base. The spectrum of N-salicylidene neomycin was found to be dependant on the ion-chamber temperature indicating that thermal decomposition plays a significant part in the fragmentation process.

The M.S. examination of neomycin trimethylsilyl ether following GLC has been reported by Murata et al¹⁵. The fragmentation pattern obtained by these workers is somewhat different to that observed by Inouye¹⁴.

It is interesting to note that the molecular ion corresponding to neamine (a major product of neomycin hydrolysis) was not observed in the mass spectrum of either the N-salicylidene or the T.M.S. derivative.

In an attempt to avoid the need for derivatisation Rinehart and Cook¹⁶ applied the technique of Field Desorption Mass Spectrometry to neomycin and successfully observed an intense response for the molecular ion at m/e 615 (M + H) together with very small peaks at m/e 455, 307 and 206 representing the loss of sugar fragments.

Table 2

 ^{13}C NMR Absorptions of hexa-N-acetyl-neomycin¹²

Carbon atom (see Fig.1)	δ ppm (from TMS)	
	Hexa-N-acetyl neomycin B	Hexa-N-acetyl neomycin C
A1	97.0	96.9
A2	54.2	54.1
A3	71.4	71.4
A4	71.4	71.4
A5	71.4	71.4
A6	40.8	40.8
D1	50.4	50.4
D2	33.2	33.1
D3	48.9	48.9
D4	76.7	76.8
D5	86.0	86.0
D6	74.5	74.5
R1	109.3	110.0
R2	74.5	73.5
R3	77.2	75.2
R4	82.4	81.8
R5	62.2	62.9
B1	98.8	97.3
B2	51.8	54.3
B3	70.3	71.7
B4	68.5	72.3
B5	74.0	71.4
B6	40.8	40.6
CH ₃	23.0 (x 3)	23.2
	22.9 (x 3)	22.8 (x 5)
CO	175.2	175.2
	174.9	175.1
	174.8	174.9 (x 2)
	174.7	174.6
	174.5	174.1
	174.1	

2.2. Physical Properties of the Solid

2.21. Hygroscopic Nature

Hirtz et al¹⁷ demonstrated the hygroscopic nature of neomycin sulphate using a thermogravimetric technique. In a more recent study Russian workers¹⁸ determined the adsorption of water by neomycin sulphate at temperatures of 23°, 90° & 100°C. Table 3 gives the results obtained at 23°C.

Table 3
Adsorption of Moisture by Neomycin
Sulphate

<u>Humidity</u> <u>%</u>	<u>Moisture adsorbed %</u> <u>(350 hours storage)</u>
0	0
10	4.15
15	5.25
25	6.90
40	10.20
60	16.70
80	38.90

2.22. Solubility

The solubilities of eleven salts of neomycin in some twenty six solvents have been reported.^{19,20,21} The values for the most commonly encountered salts are listed in Table 4.

2.23. Specific Surface Area

Using a method based on the filtration of air through a compressed layer of powder Ezerskii et al²² determined the visible specific surface area of neomycin sulphate to be 2.6 m²g⁻¹.

Table 4

Solubility of Neomycin Salts(mg/ml,corrected for solvent blank)

<u>Solvent</u>	<u>Neomycin B hydrochloride</u>	<u>Neomycin sulphate</u>	<u>Neomycin undecylenate</u>	<u>Neomycin oleate</u>	<u>Neomycin stearate</u>
Water	14.98*	6.28	0.93*	0.318	0.478
0.1N sodium hydroxide				>20	
0.1N hydrochloric acid				11.300	6.825
Methanol	5.685*	0.21	>20*	>20	17.410
Ethanol	0.61	0.055	>20*	>20	9.994
Isopropanol	0.05	0.082	>20*	>20	7.564
Isoamyl alcohol	0.285	0.202	>20	>20	11.322
Ethylene glycol	3.838*	0.033			
Propylene glycol				3.852	2.132
2-methoxy ethanol			>20*		
Benzyl alcohol	1.0*	12.35*	>20*		
Cyclohexane	0.055	0.075		>20	0.542
Cyclohexene			1.903		
Benzene	0.005	0.025	4.73	>20	0.580
Toluene	0.0	0.0	3.42		

All values \pm 0.025mg except * \pm 0.05mg.

Table 4 (Contd..)

Solubility of Neomycin Salts(mg/ml,corrected for solvent blank)

Solvent	Neomycin B <u>hydrochloride</u>	Neomycin <u>sulphate</u>	Neomycin <u>undecylenate</u>	Neomycin <u>oleate</u>	Neomycin <u>stearate</u>
Petroleum ether	0.0	0.005	2.62	>20	0.188
Isooctane	0.03		0.342	15.852	0.0
Ethyl acetate	0.03	0.028	4.70*	6.885	1.030
Isoamyl acetate	0.06	0.06	7.36*	14.080	7.014
Acetone	0.04	0.15	16.00*	9.260	2.083
Methyl ethyl ketone	0.058	0.005	5.795*	6.578	1.742
Diethyl ether	1.485*	0.07	7.989	11.288	7.328
Ethylene chloride	0.005		0.77*	3.257	0.217
Chloroform	0.06	0.0	>20*	>20	9.463
Carbon tetrachloride		0.075	7.938	>20	1.046
1,4 dioxane	0.14*	0.14*	6.485	13.038	4.500
Carbon disulphide			9.40*	>20	3.918
Pyridine	0.323*	0.923*	>20*	>20	>20
Formamide	0.45*	0.35*	>20	1.365	0.217
Dimethylsulphoxide				10.580	3.862

All values \pm 0.025mg except * \pm 0.05mg.

2.3. Physical Properties of Solutions

2.31. Density

Kigel et al¹⁸ have reported the density of 6 & 18% aqueous neomycin sulphate solutions over the temperature range 20°C to 80°C. The published values are given in Table 5.

Table 5
Densities of Neomycin Sulphate Solutions

Neomycin Concentration %	Density			
	20°C	40°C	60°C	80°C
6	1.025	1.021	1.014	1.007
18	1.086	1.081	1.076	1.068

2.32. Viscosity

Table 6 shows the reported¹⁸ viscosities for 6 and 18% aqueous neomycin sulphate solutions over the temperature range 20°C-80°C.

Table 6
Viscosity of Neomycin Sulphate Solutions

Neomycin Concentration %	Viscosity (cps)			
	20°C	40°C	60°C	80°C
6	1.25	0.86	0.64	0.623
18	1.99	1.30	0.95	0.74

2.33. Surface Tension

Kigel et al¹⁸ have reported the surface tension values of 6 & 18% aqueous neomycin sulphate solutions (Table 7).

Table 7
Surface Tension Values for Neomycin
Sulphate Solutions

Neomycin Concentration %	Surface Tension (dynes/cm)			
	20°C	40°C	60°C	80°C
6	69.81	66.00	63.30	60.30
18	72.01	68.12	65.45	62.36

2.34. Adsorption by Clays, Soils & Minerals

The adsorption of antibiotics from aqueous solution by clays and minerals has been reported by a number of authors. Pinch et al²³ concluded that, for strongly basic antibiotics such as neomycin, adsorption by clays such as montmorillonite distorted the crystal lattice of the clay 4.4Å, corresponding to monolayer adsorption. The same authors also suggested the bonding in the monolayers to be strongly electrostatic. Attempts to release the antibiotic by the addition of buffers has been demonstrated to be only partially successful²⁴. Phosphate buffers released approx. 50% of the adsorbed antibiotic whereas citrate, glycine and universal buffers were completely ineffective. In a recent study McGinity and Hill²⁵ have shown divalent magnesium ions to be more efficient than monovalent sodium ions in displacing neomycin from the negatively charged clays attapulgite and montmorillonite.

From these studies the limiting adsorptive capacities for three clays were calculated using the Langmuir equation.

Wayman et al²⁶ studied the binding of neomycin to the filtration materials celite, cellulose powder and Seitz filters. Neomycin was found to be adsorbed on all three materials. Acid-washing the cellulose powder failed to desorb all of the neomycin.

2.35. Adsorption by Ion Exchange Resins

During a study of the physico-chemical properties of some aminoglycoside antibiotics Kuzyaeva¹¹ determined the static and dynamic exchange capacities of neomycin on the cation exchange resin KB-4P-2 (a phenoxyacetic acid-formaldehyde resin) using the resin in the Na^+ form.

Kil'fin et al²⁷ have reported values for the exchange enthalpy of neomycin for three ion-exchange resins. The values of ΔH were calculated by application of the Gibbs-Helmholtz equation; the published results are tabulated below:-

Table 8
Some Exchange Enthalpies for Neomycin

<u>Ion exchange resin</u>	<u>Type</u>	<u>ΔH cal.equiv⁻¹</u>
KFU, Na^+ form	methacrylic acid-divinylbenzene	-40
KMDM6, Na^+ form	methacrylic acid-hexamethylenedimethylacrylamide	+340
KB4P2, Na^+ form	phenoxyacetic acid-formaldehyde	+570

In a further publication Kil'fin and Samsonov²⁸ reported investigations into the variation of the Selectivity Coefficient (K) with the amount of neomycin adsorbed for fourteen different ion-exchange resins. In all cases the selectivity coefficient increased with the amount of adsorbed neomycin (expressed as the % surface-coverage of the ion exchange resin), though different types of ion exchangers resulted in different responses. The degree of cross-linking of the ion exchange resin was also reported to affected the value of the selectivity coefficient. The authors concluded that poly-condensation-type ion exchange resins adsorb

neomycin more selectively than the polymerisation-type resins and that variation of the components of either type of resin had little effect on the adsorption of neomycin.

Klyueva and Gel'perin²⁹ have compared the equilibrium distribution curves for the adsorption of neomycin on ion-exchange resins from both pure solutions and typical fermentation broths.

3. Salts, Derivatives & Complexes

3.1. Salts

Many attempts to alter the physical properties of neomycin by the formation of various salts have been described. Thus the neomycin salts of the higher fatty acids, such as the stearate, palmitate and myristate^{30,31} were prepared and being water-insoluble were formulated in ointment bases. Similarly, the undecylenate salt has been prepared and processes for its production patented^{32,33,34}. The undecylenate and caprylate salts have been described as being particularly suitable as antimitotic compounds³⁵.

Various carboxylic acid salts have also been reported. Gallardo³⁶ produced the maleate salt of neomycin which, it was claimed, improved the aqueous stability of the antibiotic. A practically tasteless compound, the citrate salt, has been described by Szyska³⁸. Neomycin mandelate has been claimed to be particularly useful in the treatment of urogenital infections³⁹ while the dihydroxy-dinaphthylmethane-dicarboxylate⁴⁰ and the pamoate salts^{41,67,68} have a low intestinal absorption and are thus effective treatments for intestinal infections.

Fisher and Hall reported the propionate salt to be unsuitable for use in ophthalmic preparations³⁷. Other salts described in the literature are neomycin glucuronate⁴², succinate^{43,44}, lactate⁴⁵, ascorbate⁴⁵, phthalate⁴⁴ and the sulphosuccinate^{47,48} which has the property of improved skin penetration.

Amongst the organic sulpho acids used to prepare neomycin salts, the p-toluene sulphonate has been reported and incorporated in an aerosol spray formulation⁴⁹. Salts of neomycin with halogen substituted 8-hydroxyquinoline 5-sulphonic acids have been described as possessing low toxicity, antiseptic and antiamebic besides antimicrobial properties^{50,51}. Stankovics et al⁵² and Veciana⁵³ precipitated a complex salt of neomycin, by mixing aqueous solutions of neomycin sulphate and sodium lauryl sulphate, which had improved activity against *Staphylococcus aureus*. In a more recent investigation of this reaction, Leucuta et al¹¹³ found that the precipitated complex dissolved on addition of excess sodium lauryl sulphate. I.R. studies of the complex have indicated that amide groups, coupling reactions and H-bonding are involved in the formation of the compound. Michele and Valette have reported an enhanced antibiotic activity and a reduced toxicity for the eucalyptol sulphonate⁵⁴. Preparation of the dodecylbenzene-sulphonate⁵⁵ and the cyclohexylsulphonate⁵⁶ has also been described.

The pantothenate salt of neomycin, first described by Keller et al⁵⁷, also exhibits the property of a lower toxicity than the parent antibiotic. A further paper by the same authors⁵⁸ attributed the lower toxicity of the pantothenate to be due to a reduction in the calcium-binding ability of the antibiotic when in the form of the pantothenate salt. A similar effect has been noted by Weitnauer et al⁵⁹ with an equimolar mixture of neomycin and calcium gluconate. Numerous procedures for the preparation of the pantothenate salt have been patented^{60,61,62,63}. Abbou⁶⁴ reported the preparation of a complex salt of neomycin with a mixture of glutamic and pantothenic acids and described the compound as possessing better organoleptic properties than other neomycin salts.

Salts with the amino acids N-methylstearoylglycine⁶⁵ and glutamic acid⁶⁶, and the vitamins ascorbic acid⁶³ and nicotinic acid⁶³ have also been evaluated.

Chlorophenols are of a sufficiently acidic nature to be able to form salts with neomycin and have been reported to possess both antibacterial and mycocidal properties^{69,70,71}. Shaw⁷⁰ has described the topical use of such a compound, neomycin hexakis(pentachlorophenyl)phosphate, in the treatment of dental root infection and periodontic cavities.

A salt formed by reacting neomycin with p-amino benzene sulphonylacetamide was described by two groups of workers^{72,73}. The salt combined the chemotherapeutic properties of the components. Another unusual salt of neomycin described in the literature is that with m-sulphonylbenzaldehyde isonicotinoylhydrazide^{78,79}. The compound is reputed to be less toxic than neomycin and to be more active against tubercle bacilli than the individual constituents.

Salts of neomycin with the inorganic acids have also been described. The sulphate salt is the usual commercial form in use today but neomycin borate has been used in ophthalmic preparations⁴⁶.

Not all neomycin salts, however, possess advantages such as a reduced toxicity. In fact, the orotic acid(1,2,3,6-tetrahydro-2,6-dioxo-4-pyrimidine carboxylic acid) salt was reported to have a greater toxicity than neomycin itself^{74,75}. Some uncertainty of this fact is apparent as other authors have reported the two compounds to be of a similar toxicity⁷⁶. The salt of neomycin with usnic acid(an antibacterial substance found in lichens) has been reported⁷⁷ to have less antibacterial activity than the neomycin used in the preparation of the salt.

3.2. Derivatives

The neomycin molecule contains both free amino and free hydroxy groups. Many workers have exploited the possibility of chemical derivatisation at these positions in attempts to reduce the toxicity of the parent antibiotic. Substitution of methane sulphonate groups at the amino nitrogen has been reported by Umezawa et al^{83,84} and by Boissier et al⁸⁵. Both groups of workers described the derivative to be less toxic

than the parent antibiotic. Boissier claimed that the 'in vitro' and 'in vivo' antibacterial activity of the neomycin is preserved following derivatisation. However, Umezawa, working with fradiomycin, described the N-methyl sulphonate derivative as having half the activity of fradiomycin when measured against gram-positive bacteria and only one tenth the activity when measured against gram-negative bacteria. Russian workers⁸⁹ have reported the reduction in antibacterial activity to be proportional to the degree of N-substitution. Alternative procedures for the preparation of N-methyl sulphonyl neomycin have been reported by a number of authors^{86,87,88} and the pharmacology of the compound has been extensively investigated by Di Marco and Bertazzoi⁹⁰. The preparation of the following sulpho derivatives has also been described:

N-methylsulphino neomycin^{87,91}
N-phenylsulphonyl neomycin⁹³
N-acetamidophenylsulphonyl neomycin⁹³
N-p-aminophenylsulphonyl neomycin⁹³
N-trichloromethylthio neomycin⁹²
N-ethylthiocarbonyl neomycin⁹³
and neomycin N-sulphonate⁹⁴

Vanderhaeghe⁸⁰ has studied the alkylation of neomycin. A reduction in toxicity was noted following alkylation but this was coupled with a complete loss of antibacterial properties. The resulting compound, however, was found to possess good hypocholesteremic activity. The effect of N-alkylation, O-alkylation, N-acylation and O-acylation has been investigated by Magyar et al⁸¹. These workers reported the loss of biological activity to be proportional to the number of groups substituted. Further investigations by these workers demonstrated that conversion of amino groups into quaternary ammonium groups resulted in the production of compounds having a mild tuberculostatic effect. Penasse et al⁸² have described an alternative procedure for the preparation of N-alkyl derivatives.

3.3. Complexes

The formation of a zinc-neomycin complex has been reported by Chornock⁹⁵ and by Keller and Cosar⁹⁶. Electrophoresis was used to demonstrate the formation of a complex which was described as possessing a similar biological activity to neomycin but a decreased toxicity. More recently Agrawal, Harmalker and Vijayawargiya⁹⁷ have described the formation of a copper-neomycin complex. With a stoichiometry of 1:1, the blue coloured complex has been made the basis of a spectrophotometric assay method for neomycin (See Section 6.26).

The complexation of neomycin with a number of biochemically important compounds has been reported. Interaction of the antibiotic with heparin was first investigated by Higginbotham and Dougherty⁹⁸. Using a spectrophotometric procedure, which measured the amount of dye released from a toluidine blue-heparin complex on addition of neomycin solution, these authors studied a number of antibiotics and their reaction with heparin. Gubernieva and Silaev⁹⁹ employed electrophoresis to investigate the nature of the neomycin-heparin complex and suggested the compound may be more than a simple cation-anion interaction. The possibility of additional H-bonding following the initial ionic interaction was postulated. A more detailed study of the complexation of neomycin with heparin, DNA, RNA and polyphlorethin phosphate has been described by Hein¹⁰⁰. The complexes were prepared by mixing aqueous solutions of neomycin sulphate and the complexing agent under investigation. The precipitate which formed redissolved on addition of sodium hydroxide solution or aqueous solutions of silver nitrate, manganese sulphate and cobalt chloride. Subjection of the precipitated complex to an agar diffusion experiment demonstrated the compound to be biologically active.

Using an agarose gel system Kunin and Tupasi¹⁰¹ observed the formation of a precipitation band between zones of dextran sulphate and neomycin. The authors attributed the precipitation to be a result of complex formation

and not the result of an antigen-antibody interaction which the system is usually employed to detect.

Harris¹⁰² has studied the complexation of neomycin with pectin and demonstrated the inhibition of complex formation in the presence of an electrolyte. Potentiometric measurements indicate the mechanism of the reaction to be a cation-anion interaction. H-bonding between the hydroxy groups of pectin and sugar moieties of neomycin has been suggested and would further stabilise the compound.

The complexation of neomycin with anionic dyes such as amaranth (F.D. & C. Red No.2) is well known^{100,102} and has been made the basis of a quantitative assay for neomycin^{102,25}. These complexes are again of the cation/anion type and their formation is dependant on the ionic strength of the solution.

Inorganic condensed phosphates have been reported to complex with neomycin and in a study of these compounds, Singh¹⁰³ demonstrated the strength of the complex to be dependant on the degree of polymerisation of the phosphate. By increasing the number of phosphate units in the polymer over the range 1 to 16 units an increasing strength of complexation was observed. Beyond this upper limit, however, the strength of the complex remained constant. Relative complex-strengths were assessed by titrimetry with potassium chloride solution.

A number of workers have utilised electrophoresis to establish the formation of a complex between neomycin and the proteins in blood-serum and plasma^{104,105,106,107,108,109,110,111}. However, the nature of the complex is still unknown. Geitman has extensively studied the phenomena of protein-binding of antibiotics^{111,112}. In a paper published jointly with Kivman¹¹², Geitman reported that neomycin did not bind to any of the protein fractions when added to serum but bound to the isolated albumin and globulin fractions. An earlier paper¹¹¹ described the globulin-neomycin complex to be of greater stabil-

ity than the albumin-neomycin complex and established 1mg of serum protein to bind 0.06 units of neomycin.

4. Synthesis and Production

4.1. Commercial Biosynthesis

Waksman et al²⁸⁶, in 1949, first reported the production of neomycin by fermentation of a culture of *S. fradiae* (3535). The same organism subsequently formed the basis of an industrial fermentation process for the biosynthesis of neomycin^{287,288}. Isolation of the antibiotic from the fermentation media is accomplished by use of ion-exchange resins, such as Amberlite IRC 50^{225,250,251}.

Following the original report by Waksman, a number of other authors have described *S. fradiae* to yield antibiotics on fermentation. The resulting substances were named streptothricin BI²⁸⁹, mycerin²⁹⁰ and colimycin²⁹¹, but have since been shown to be identical to the neomycin complex.

4.2. Synthesis of Radio-Labelled Neomycin

The biosynthetic preparation of ¹⁴C labelled neomycin was first described by Sebek²⁶⁷ who studied a number of substrates for this purpose but concluded ¹⁴C-labelled glucose was the most satisfactory. Although 68% of the radio nuclide was lost as respiratory ¹⁴CO₂, 19% of the total activity added prior to fermentation was incorporated into the neomycin sulphate. This method of preparing ¹⁴C-labelled neomycin has been extensively used to study the mechanism of neomycin biosynthesis. Stroshane¹³ has described the synthesis of ¹³C-neomycin and ¹⁵N-neomycin, again as part of an investigation of neomycin biosynthesis. ¹³C-glucose served as precursor for ¹³C-neomycin and glucosamine labelled with ¹⁵N was utilised for the preparation of ¹⁵N-neomycin.

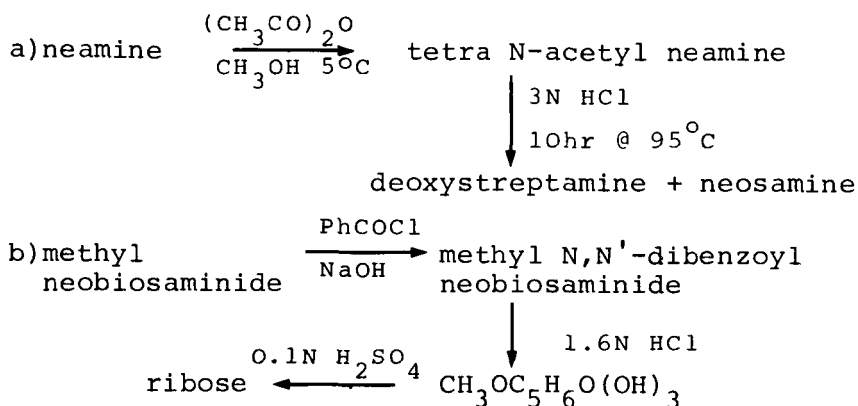
A procedure for labelling neomycin with tritium has been described by Jackson et

at²⁹² and involves passing an electric discharge across a vessel containing tritium and neomycin sulphate. Labile tritium was removed by washing with a polar solvent and the tritium-labelled neomycin purified by multiple recrystallisation.

5. Stability and Degradation

5.1. Hydrolytic Degradation

The degradation of neomycin has been extensively studied by Rinehart¹ as a means of establishing the structures of the neomycin components. Fig. 5 illustrates the route by which complete degradation of the antibiotic was achieved. Extremely vigorous conditions are necessary for the hydrolysis of neamine as this compound is resistant to acid-hydrolysis. However, under these conditions the chemical entities neosamine C (from neamine) and ribose are not observed because vigorous hydrolysis immediately decomposes the compounds further. By derivatising the amino groups of the neamine and neobiosaminide molecules Rinehart was able to lower the resistance of these compounds to hydrolysis and thus use mild hydrolytic conditions to successfully isolate neosamine C and ribose:



Russian workers¹⁹⁸ attempted to prepare the degradation products in a pure form by carrying out the hydrolysis in the presence of an ion exchange resin which adsorbed the intermediate

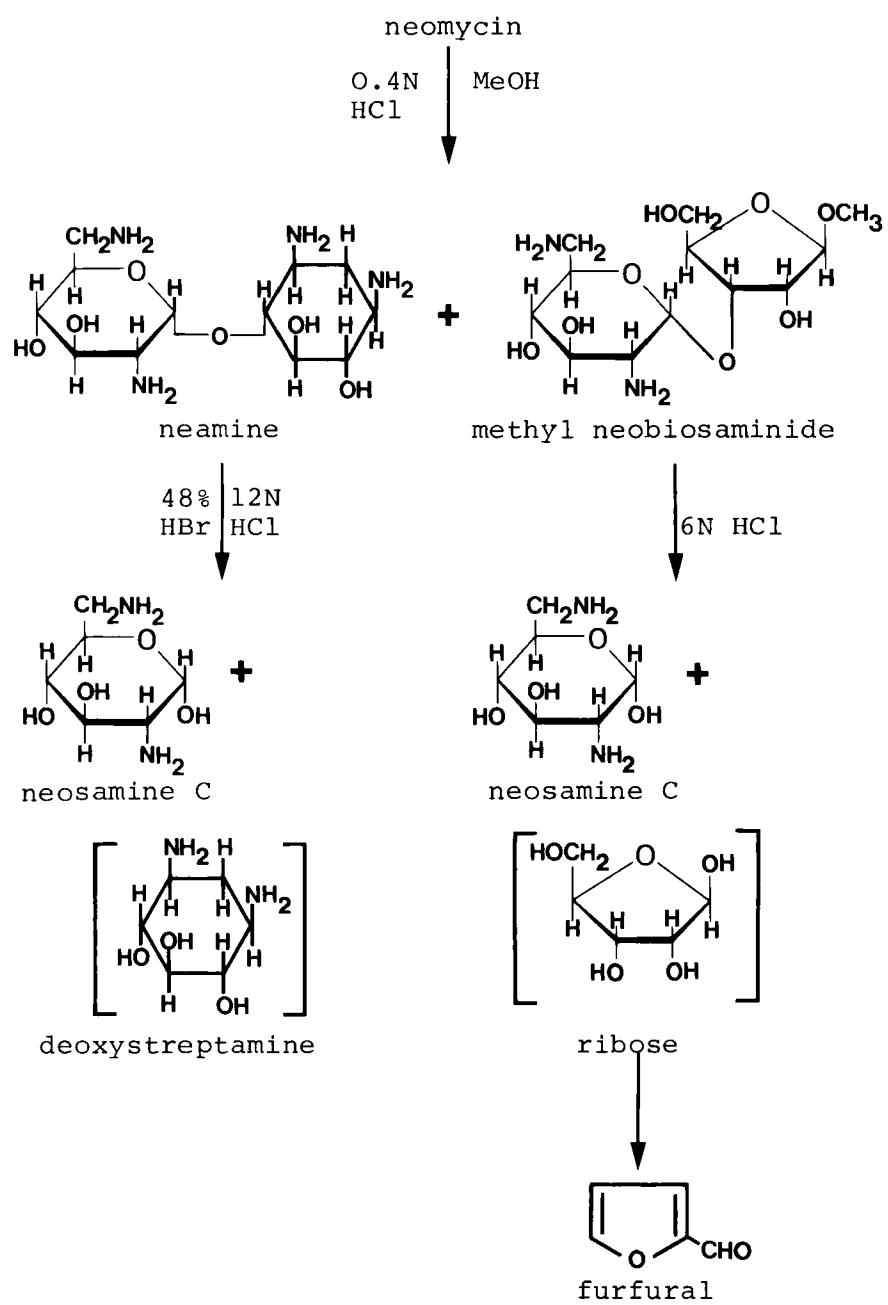


Figure 5. Hydrolytic degradation of neomycin C

hydrolysis products, thereby preventing further decomposition. The degradation products were released from the resin by gradient-elution.

5.2. Stability of Bulk Material

Japanese workers¹¹⁵ have described the stability of bulk fradiomycin (neomycin B) sulphate which was shown to have retained 99% of its microbiological potency after storage for 24 months. Neomycin sulphate powder has been reported to be stable for at least 3 years at 20°C^{298,305}.

Neomycin sulphate may also be heated at 110°C for 10 hours, during dry-heat sterilisation, without loss of potency, though some degree of yellowing is apparent²⁹⁸.

5.3. Stability in Aqueous Solution

Swart et al have demonstrated the stability of neomycin hydrochloride in aqueous solution over the pH range 2-9²⁹³. Further investigations by Simone and Popino²⁹⁸ confirmed the aqueous stability at 23°C but at 45°C potency losses of up to 94% were noted over a period of 2 years with solutions in the pH range 4-8.

Leach et al²⁹⁴ described a purified neomycin to be stable to the action of alkali but not to acids. Refluxing the antibiotic with barium hydroxide for a period of eighteen hours failed to show a loss in microbiological potency.

Japanese workers have described a stable aqueous solution of neomycin with the incorporation of a borate buffer (pH 6) and E.D.T.A.²⁹⁵ The presence of 1-10% of glycerol, propylene glycol or mannitol has been claimed to improve the solution appearance by preventing discoloration²⁹⁶. The presence of polyols also prevented a decrease in pH value of the solution. Discoloration may also be prevented by addition of 0.1% sodium metabisulphite at a solution pH of 6.6 to 6.8²⁹⁷.

5.4. Stability in Pharmaceutical Formulations

Numerous reports concerning the stability of neomycin in various dosage forms have been published. Simone and Popino²⁹⁸ studied the stability of neomycin in liquid dosage forms such as nasal drops, mouth washes and tinctures. The antibiotic was stable in all the formulations tested, except Dobells solution (a mouth wash), for at least 6 months at 20°C. Some formulations were stable for considerably longer.

Heyd²⁹⁹ has studied the stability of neomycin in aqueous gels and formulated a satisfactory product by adsorbing the antibiotic on an ion exchange resin, Amberlite IRP-69M, prior to incorporation in the gel.

Reconstituted aqueous suspensions of neomycin sulphate, containing tragacanth and sugar have been shown to be stable for 10 days when stored at 4°C but some loss of potency was observed when the suspension was exposed to daylight³⁰⁰. Non-aqueous suspensions containing peanut oil and lanolin have been reported²⁹⁸ which are stable for 1 year at 20°C.

Simone and Popino²⁹⁸ have considered the stability of neomycin in both hydrophobic and hydrophilic ointment bases. No loss of potency over a period of 1 year at 20°C was reported for formulations containing carboxymethylcellulose, polyethylene glycol (P.E.G.) or white-soft paraffin. However, formulations containing hydrous lanolin were reported to be unstable. All materials used in the formulations were obtained from U.S. sources. Coates et al³⁰¹ investigated the use of P.E.G. from British sources and described neomycin as being incompatible with the materials tested.

Solid dosage forms of neomycin as tablets and troches have been prepared²⁹⁸ and are reported to be stable at 20°C and at 56°C.

In the area of animal health nutrition a feed-premix containing neomycin and oxy-tetracycline has been described. With neomycin

at a level of 20g/lb the product is stable for 1 year at 20°C, though an 8% loss of potency was observed on storage for 6 months at 38°C³⁰⁵. A medicated drinking-water containing 100 ppm of neomycin and prepared with tap-water, showed no significant loss of potency after 48 hours storage at 38°C in either glass containers or galvanised troughs³⁰⁵.

5.5. Compatibility with Excipient Materials

The reaction of neomycin with many compounds has been described in Section 3, hence numerous reports of neomycin incompatibility may be expected. Dale and Rundman³⁰⁴ have extensively reviewed the compatibility of neomycin with substances that may be encountered by the formulation pharmacist. Kudalker et al³⁰³ have described the incompatibility of the antibiotic with rancid oils, and the incompatibility with bentonite, a montmorillonite clay, has been reported by Danti and Guth³⁰⁶. The incompatibility with lactose, causing a discoloration of the mixture has been studied by Hammouda and Salakawy³⁰⁷. The amount of browning produced was shown to be dependant on the initial pH of the solution. The rate of discoloration of the lactose/neomycin powder was directly related to the temperature of storage and the relative humidity of the atmosphere. Discoloration was overcome by addition of sodium bisulphite.

Florestano et al³⁰⁸ compared the release of neomycin from P.E.G. diester ointment bases and grease-type bases and concluded the former to be preferred though Coates et al³⁰¹ have described P.E.G. from British sources to be incompatible with neomycin.

5.6. Compatibility with Other Actives

Neomycin forms an insoluble complex when added to aqueous solutions of corticosteroid phosphates. To overcome this incompatibility, McGinty and Brown³⁰⁴ incorporated disodium hydrogen phosphate in the ophthalmic formulation. The mechanism by which the complexation is prevented has not yet been fully determined.

6. Methods of Analysis

6.1. Identification

Table 9 lists a number of colorimetric tests which have been used as identity tests for neomycin. No one test, however, has been demonstrated to be specific for neomycin and it is thus also advisable to establish the absence of other chemically similar antibiotics by suitable means.

Table 9
Colorimetric Identity Tests for
Neomycin

<u>Test</u>	<u>Chemical group- ing utilised in reaction</u>	<u>Colour obtained</u>	<u>Reference</u>
Glucosamine	glycoside	cherry	114
Molisch	pentose	purple	115
Ninhydrin	amino	purple	115,116
Furfural	ribose	pink-red	116
Phloro- glucinol	ribose	pink-red	117

Both thin-layer and paper chromatography will provide specific identification of neomycin. Numerous systems for this purpose will be found listed in section 6.34.

In addition to the above chemical tests a number of microbiological procedures have been reported. Heinmann et al¹¹⁸ have described a simple test to differentiate neomycin-like molecules from tetracyclines and chloramphenicol. When added to a column of inoculated agar only the neomycin-like molecules show an area of growth-inhibition at the top of the column.

A more specific microbiological procedure has been reported by Freres and Bulland¹¹⁹. By using four test organisms (*Bacillus cereus*, *Bacillus subtilis*, *Sarcina lutea* and *Micrococcus flavus*) patterns of inhibition were determined for each of the 12 antibiotics extracted into three solvents. Comparing the inhibition pattern of an

unknown antibiotic with the standard patterns, the authors were able to identify the presence of individual substances in animal tissue. A similar test has also been described by Tiecco et al¹²⁰.

6.2. Chemical Procedures

6.21. Titrimetric Assay

The determination of neomycin by non-aqueous titration has been described by Penau et al¹²¹. Neomycin base is allowed to react with standardised perchloric acid; the excess acid is then back-titrated with potassium hydrogen phthalate using crystal violet as indicator. To determine the neomycin content of the sulphate salt the same authors precipitated the sulphate with benzidine before reacting the neomycin with perchloric acid. The amount of benzidine required to precipitate the sulphate is calculated from the sulphate content which is itself determined by titration with sodium hydroxide.

An alternative method for determining the sulphate content of neomycin sulphate has been reported by Roets and Vanderhaeghe¹²². The procedure involves a titration with barium chloride and must be carried out in a 30-40% solution of alcohol in order to obtain a sharp end-point. As the antibiotic is insoluble in alcohol it is necessary to remove the neomycin part of the molecule prior to titration. This is accomplished using a cation exchange resin such as Dowex 50-X8.

During an investigation of the complexation properties of neomycin, Harris¹⁰² reported the titration of neomycin conductometrically with amaranth (F.D. and C Red No. 2). The end-point of the titration is sharp and has good reproducibility.

6.22. Polarography

The use of differential pulse polarography to determine neomycin sulphate in aqueous solutions has been described by Siegeman et al¹²³. Following acid hydrolysis of neomycin, the solution was adjusted to pH 4 and 0.1M acetate

buffer (pH 4) added as supporting electrolyte. Reduction potentials of -0.19 and -0.36V were obtained. The above authors also described the application of this technique to the examination of samples of skin previously treated with neomycin-containing formulations.

6.23. Polarimetry

Neomycins B and C have been shown to differ in their specific rotation values, neomycin B having a specific rotation of $+80^{\circ}$ and neomycin C a specific rotation of $+120^{\circ}$ ^{124,127}. Brooks et al¹²⁵ made this fact the basis for a number of methods to determine the B & C content of commercial neomycin. The specific rotation of the test solution is determined at 25°C and total neomycin determined either titrimetrically or spectrophotometrically. By substitution of these values in a suitable equation the concentration of neomycins B and C are calculated. In a second method the same authors determined the specific rotation of the test-solution at temperatures of 25° and 75°C . The change in the value of specific rotation on increasing the temperature from 25° to 75°C can then be used to calculate the amounts of neomycin B and C in the sample.

The use of an automatic polarimeter with a flow-cell has been reported by de Rossi¹²⁶, to monitor the eluate from an ion-exchange column (Bio-Rad AG1-X2) through which a solution of neomycin was passed. The detection of an optically active substance was recorded electronically with a suitable pen recorder. By determining the areas of the peaks recorded, the amounts of neomycins B and C and neamine in a number of commercial samples have been determined.

6.24. Radio-chemical Assay

To determine the relative amounts of neomycins B, C and neamine by a radio chemical method, Kaiser¹²⁸ separated the ^{14}C -labelled N-acetyl derivatives by paper chromatography and quantitated the chromatograms by liquid scintillation counting. A coefficient of variation of 3.6% was obtained.

Seaman and Stewart¹²⁹ have described a radio-chemical assay for determining neomycin on cotton-fabric. Neomycin is reacted with carbon disulphide forming a dithiocarbonate which is then decomposed with [¹¹⁰Ag] silver nitrate. The precipitated [¹¹⁰Ag] silver sulphide, which is directly related to the amount of neomycin present, is estimated by counting.

Recently an isotope dilution method has been reported¹³⁰ for assaying neomycin sulphate. However, it is first necessary to prepare ¹⁴C-labelled neomycin sulphate. This is accomplished by adding ¹⁴C-labelled glucose to a small-scale fermentation of *S. grisea*. ¹⁴C-labelled neomycin can then be extracted by solvent-extraction or by ion-exchange chromatography.

6.25. Fluorimetric Assay

The neomycin molecule does not exhibit fluorescence but following suitable derivatisation two spectrofluorimetric determinations have been described. Maeda et al¹³¹ reported the complexation of simple hexosamines with pyridoxal and zinc ions to result in a fluorescent derivative. Applying this procedure to neomycin, Simpson¹³² demonstrated a linear response over the concentration range 0-50 µg/ml of neomycin sulphate.

The reaction of neomycin with fluorecamine to form a fluorescent complex has been reported by Kushnir & Barna¹³³. The fluorescence intensity varies with both the pH of the solution (optimum pH range 7.5-9.5) and the amount of fluorecamine added. This procedure may be used to determine neomycin at very low levels, the minimum concentration determinable being 45 ng/ml.

6.26. Spectrophotometric Assay

Spectrophotometric assays of neomycin may be conveniently divided into two groups:-

- (a) Direct methods, involving the intact neomycin molecule.
- (b) Indirect methods, involving hydrolysis of neomycin prior to assay.

(a) Direct Methods

O'Keefe and Russo-Alesi¹³⁴ first reported the application of ninhydrin to the assay of neomycin, the procedure being an adaptation of that described by Moore and Stein¹³⁵ for the assay of amino acids and involving measurement at 570nm of the purple-coloured complex formed. Maehr and Shaffner¹³⁶ have applied this procedure to the determination of neomycin in chromatography - column eluates. Gerosa and Melandri¹³⁷ attempted to apply the Moore and Stein method to the quantitative determination of neomycin in pharmaceutical preparations but reported a linear response only over the short concentration range of 400-600µg/ml of neomycin. Borowiecka¹³⁸ and Thorburn-Burns et al¹³⁹ however, reported no such limitation and successfully applied the method to the assay of neomycin in pharmaceutical formulations. Pregnatalto and Sabino²⁰⁰ incorporated glycerine into the ninhydrin solution and reported an improved sensitivity and reproducibility enabling neomycin concentrations as low as 4 µg/ml to be determined. An automated ninhydrin assay has been described by Kaptionak, Biernacka and Pazdera¹⁴⁰ based on a modified Moore and Stein method¹⁴¹. An alternative reagent which also reacts with the primary amino groups of neomycin has recently been described¹⁷². The reagent, diclone (2,3-dichloro-1, 4-naphthoquinone), reacts with neomycin base to form an orange-coloured product when the solution is adjusted to pH 4.

Complexation of neomycin with various dyes was reported by Hein¹⁰⁰ and the aqueous insolubility of the complex with amaranth (F.D. & C Red No. 2) has been utilised by Hill²⁵ and by Bufton and Saddler¹⁴² to assay neomycin in aqueous solution. Amaranth may also be used to determine neomycin in production samples from fermentation-recovery¹⁶⁸. The dye, Orange II, has been similarly¹⁴³ described (λ max. = 484 nm). Complex formation between neomycin and the dye is very dependant on the ionic strength of the solution, thus necessitating careful control of reaction conditions to ensure complete precipitation of the complex during the assay procedure. Reaction of sodium 1,2-naphtho-quinone-4-sulphonate with

neomycin¹⁴⁴ produces an orange/yellow product, λ_{max} 460nm in acetic acid, which has been utilised to determine the neomycin content of ophthalmic solutions giving results in good agreement with microbiological assays.

Strongly basic antibiotics may be precipitated by formation of the coloured reineckate salt which may then be determined spectrophotometrically¹⁶⁵. Bickford¹⁶⁶ dissolved the precipitated neomycin reineckate in acetone and has successfully used this procedure to assay neomycin extracted from topical formulations. Roushdi et al¹⁷³ preferred to oxidise the precipitate with potassium permanganate and then colorimetrically estimate the chromate produced with diphenylcarbazide.

Aromatic aldehydes react with primary amines forming Schiff's bases which are often coloured. The colour may then be used to quantitate the amine. Using this principle Kocy¹⁶⁷ has shown neomycin to form a yellow-coloured Schiff's base with salicylaldehyde though the quantitative aspect of this procedure is, as yet, incomplete.

The complexation of neomycin with copper results in the formation of a blue-coloured compound which has also been made the basis of a colorimetric determination of neomycin in pharmaceutical formulations⁹⁷. Maximum colour intensity was observed at a solution pH of 10. Further investigation showed the stoichiometry of the complex to be 1:1 in alkaline solution.

(b) Indirect Methods

Hydrolysis products of neomycin may be an amino-sugar, a pentose or furfural depending on the reaction conditions chosen. Each of these entities has been utilised for indirect spectrophotometric determination of neomycin.

The presence of a sugar moiety in neomycin was demonstrated by Hamre et al¹⁴⁵ who used carbazole and anthrone to quantitate neomycins B and C in commercial neomycin. The procedures employed were those previously applied to manosido-streptomycin^{146,147}. Penau et al¹⁵¹ reported poor

agreement between results obtained by the anthrone assay and microbiological procedures and suggested the presence of carbohydrate impurities in neomycin to be the reason.

Other reagents which form coloured complexes with sugars such as orcinol^{148,149,150} (3,5-dihydroxytoluene) and phloroglucinol¹³⁷ (1,3,5-trihydroxybenzene) have been reported for the spectrophotometric assay of neomycin. Hoodless claimed the phloroglucinol procedure to be less time-consuming than other methods¹¹⁷ and applied this procedure to neomycin sulphate raw material. Doulakas¹⁷⁴ adopted a similar procedure to determine the neomycin sulphate content of ophthalmic ointments. Interfering substances co-extracted from the ointment were separated from neomycin by TLC before reacting the antibiotic with phloroglucinol.

The orcinol procedure has been used to assay the neomycin content of fermenter broths^{148,149,150} though it is necessary to first separate the antibiotic by ion-exchange chromatography. Korchegin¹⁵⁰ reported good agreement between the orcinol assay and the microbiological assay of production samples. Khromov and Starunova¹⁷⁵ and Korchagin et al¹⁶⁹ compared the orcinol and microbiological assays for determining the neomycin content of polymer-films and, again, good agreement between the two procedures was noted.

After mild hydrolysis, neomycin has been shown to give a positive Elson-Morgan reaction¹⁵¹, a reaction characteristic of amino-sugars¹⁵². A method involving this reaction has been made the basis of a quantitative assay for neomycin¹⁵¹ which has been used to determine the neomycin content of fermentation broths¹⁵⁴.

Vigorous hydrolysis of neomycin results in the formation of furfural¹⁵⁴ which may be quantitated in a number of different ways^{155,156}. Dutcher et al¹⁵⁷ used a UV spectrophotometric procedure, measuring the absorbance of the solution at 280nm. A similar procedure combined with measurement of the optical rotation has been used by Brooks et al¹⁵⁸ to determine the neomycin B and C content

of the antibiotic. Colorimetrically, the furfural obtained from neomycin may be assayed with an aniline by measuring the amount of pink/red complex formed. Morgan et al¹⁵⁹ used 4-bromoaniline in this context, the 4-bromo derivative being more resistant to the formation of interfering coloured products than aniline itself¹⁶⁰. This method has been applied to various pharmaceutical formulations and results show good agreement with microbiological assays. Aniline has been used to determine the neomycin content of fermenter broths and other production samples¹⁶¹, neomycin undecylenate and neomycin caprylate¹⁶².

Much work has been carried out to establish optimum conditions for a reproducible conversion of neomycin to furfural, quantitative conversion of the pentose moiety to furfural being difficult and the procedures tedious^{163,164}. The acid strength for optimum production of furfural must be such that it is strong enough to hydrolyse the sugar molecule, yet insufficiently strong to decompose the furfural formed. A review of the literature by Ivashkiv¹⁷⁰ showed 12% hydrochloric acid to be accepted as optimum for conversion of pentoses to furfural. However, as neomycin must first be decomposed to the pentose before hydrolysis of the sugar can take place, more vigorous conditions are required. Dutcher et al¹⁵⁴ used 40% sulphuric acid to produce an acid concentration of 37% in the solution for hydrolysis and obtained maximum formation of furfural after 1½ hrs reflux. Morgan et al¹⁵⁹ however, used 70% sulphuric acid to produce a 25% acid concentration in the hydrolysis solution and obtained maximum formation of furfural after one hour. Ivashkiv¹⁷⁰ has evaluated the use of both hydrochloric and sulphuric acids and found up to four times more furfural to be produced by sulphuric acid, the yield being extremely sensitive to the concentration of acid employed. Continuing further work with sulphuric acid, the formation of furfural as a function of neomycin concentration was examined and the conclusion reached that better yields of furfural are obtained at low concentrations of the antibiotic. Heyes¹⁷¹ has shown a constant amount of furfural to be produced on hydrolysis with 27-28% sulphuric acid, refluxing for 1 hour. This makes it unnecessary to accurately

pipette concentrated solutions of sulphuric acid.

From the above discussion it would, therefore appear necessary to establish optimum conditions for furfural production in each individual case.

6.3. Chromatographic Procedures

6.31. Counter-Current Distribution

Swart, Lechevalier and Waksman¹⁷⁶ have examined neomycin by the method of counter-current distribution using a partition-system of borate buffer (pH 7.6)/pentasol (synthetic amyl alcohol)/stearic acid. Three major components with varying amounts of neamine were found in samples of neomycin B from various sources. Determination of the amount of antibiotic remaining in each tube following partition was carried out microbiologically. In an extension of this study to the whole group of neomycin-like molecules Schaffner¹⁷⁷ however, has been unable to separate neomycin B into the separate components reported by Swart et al¹⁷⁶ using either of four partition systems. The systems employed were:-

- Systems 1-3) 0.5M borate buffer at pH 7.3,
7.6 or 7.8/pentasol/stearic acid.
- 4) 0.5% bicarbonate buffer/pentasol/
stearic acid.

Both chemical and microbiological assays were utilized to determine the distribution of antibiotic in the tubes. In all experiments the microbiological assay results were more erratic than the corresponding chemical assays and on this basis Schaffner explained the difference between his results and those of Swart et al¹⁷⁶ who used only microbiological determinations. In this same work Schaffner was able to demonstrate the same distribution pattern for both framycetin and neomycins B and C and thus tentatively identified framycetin as neomycin. Similarly, but using a partition system of 3% p-toluene sulphonic acid in 1N acetate buffer (pH 3.8)/butanol Baikina et al¹⁷⁸ have identified mycerin and colimycin as neomycin and Sannikov¹⁷⁹ has identified framycin with neomycin.

From a preparative aspect, Peck et al¹⁸⁰ have employed the counter-current distribution technique with a partition system of water/butanol/p-toluene sulphonic acid to separate and purify the neamine (neomycin A) present in commercial neomycin.

6.32 Electrophoresis

Neomycin is a readily ionisable molecule and should thus be separable from other antibiotics by application of an electric field (zone electrophoresis). Various workers have successfully applied this technique to neomycin and Table 10 summarises the conditions reported in the literature. A number of authors described the qualitative separation of neomycin from other chemical-types of antibiotics using paper-electrophoresis^{181,185,187} while Ochab¹⁸⁹ described systems specifically designed to separate compounds within the aminoglycoside group of antibiotics. Carr et al¹⁸⁶ have reported the quantitative determination of neomycin in the presence of polymyxin and bacitracin. Using paper electrophoresis quantitation of neomycin was accomplished colorimetrically with ninhydrin.

Lightbown and DeRossi¹⁸⁸ substituted an agar-gel support for the more usual chromatography-paper and successfully separated neomycin from a variety of antibiotics but not from paromomycin. However, quantitation of this procedure was not possible due to the elongated zone of inhibition produced by neomycin on incubation of the agar-gel. The same authors also reported some anomalous behaviour of the neomycin ion in the presence of certain types of agar. Neomycin is a basic molecule and under normal circumstances would be expected to behave as a cation and migrate to the anode. However, with certain types of agar the neomycin was observed to migrate towards the cathode. This phenomena was repeatedly confirmed though not explained. A system suitable for the quantitative estimation of neomycin using an agar-gel support has been described by Grynne¹⁸³. To obtain a good clear zone for neomycin it was necessary to change the buffered electrolyte/agar-gel to a phosphate system at pH 6.5. Detection of the

Table 10
Electrophoresis of Neomycin

<u>Electrolyte</u>	<u>Medium</u>	<u>Conditions</u>	<u>Detecting Agent</u> (See also Section 6.34)	<u>Reference</u>
1. Veronal sodium buffer, pH 8.6	paper	15.3v/cm	ninhydrin	181
0.07M barbital buffer, pH 8.6	cellulose acetate	200v for 2 hours	Ponceau S.	182
Veronal buffer, pH 8.6	paper	25v/cm	<i>B.subtilis</i> ATCC 663	183,184
2. 5% formic acid(pH 2)	paper	15.3v/cm	ninhydrin	181
3. Acetic acid/pyridine/ water(2:6:92)	paper	225v for 6 hours	ninhydrin;aniline	185
(0.25:7.5:92.25)	cellulose acetate	400v for 1 hour	ninhydrin;Ponceau S	186
(20:6:9.74)	paper	225v for 6 hours	ninhydrin	187
4. Acetic acid/formic acid/water(6:3:91)	paper	700v	ninhydrin,Ponceau S	186

Table 10(Contd...)
Electrophoresis of Neomycin

<u>Electrolyte</u>	<u>Medium</u>	<u>Conditions</u>	<u>Detecting Agent</u> (See also Section 6.34)	<u>Reference</u>
5. Trishydroxymethylamino- methane/maleic acid/ sodium hydroxide (0.907%:0.870%:0.002%) pH 5.6	agar-gel buffered, pH 5.6	2000v, 200 mA	<i>B.subtilis</i> NCTC 8241	188
6. 2M formic acid/0.1M toluene sulphonate/ propanol/water (50:50:5:40)pH 1.8 (40:5:5:50) pH 1.9	paper	340v	<i>B.subtilis</i> ATCC 6633	189
7. 2M formic acid/0.1M toluene sulphonate/ water(40:5:55)pH 1.9	paper	340v	<i>B.subtilis</i> ATCC 6633	189
8. 1M ammonium hydroxide/ 1M sodium hydroxide/ 0.1M toluene sulphonate/ propanol/water (20:0.5:10:10:69.5) pH 10.8	paper	340v	<i>B.subtilis</i> ATCC 6633	189

Table 10(Contd...)

Electrophoresis of Neomycin

<u>Electrolyte</u>	<u>Medium</u>	<u>Conditions</u>	<u>Detecting Agent</u> (See also Section 6.34)	<u>Reference</u>
9. 1M ammonium hydroxide/ 1M sodium hydroxide/water (20:0.25:79.75)pH 11.5	paper	340v	<i>B.subtilis</i> ATCC 6633	189
10. 1M sodium hydroxide/ water(5:95)pH 12.2	paper	340v	<i>B.subtilis</i> ATCC 6633	189
11. Acrylamide gel , pH 4.6	acrylamide gel	100v, 2mA	Naphthalene Black	190
12. pH 6.5 phosphate buffer (KHPO ₄ ,22g/l; KH ₂ PO ₄ ,28g/l)diluted 1:40 with distilled water.	agar-gel buffered at pH 6.5	310 to 360v (current ‡ 50mA)	<i>Staphalococcus</i> <i>epidermidis</i> ATCC 12228	183

antibiotic was achieved bioautographically. The organism *Staphalococcus epidermidis* ATCC 12228 was found to be the most sensitive for this purpose. (The description of alternative bioautographical systems will be found in section 6.34.)

Using an acrylamide-gel as support, Coombe¹⁹⁰ has demonstrated the quantitative assay of neomycin with a recovery of 96% in the presence of bacitracin and polymixin. In this case quantitation was achieved by densitometry after staining the gel with naphthalene black.

Electrophoresis has also been employed to separate neomycin from analytically-interfering substances such as proteins. Hence Brammer and Hemson¹⁸² have determined the neomycin content of blood serum. Neomycin was separated from the serum proteins by electrophoresis on cellulose acetate and assayed colorimetrically following elution from the support.

A modified technique consisting of an electric field applied perpendicular to a flowing buffer solution and using chromatography paper as support (cross-electrophoresis) has been exploited to study the reaction of neomycin with heparin^{99,184}. Evidence for the formation of a neomycin-heparin complex was obtained by this means.

6.33. Column Chromatography

The column chromatographic separation of neomycin B, neomycin C and neamine on an ion-exchange column has been described by Maehr and Schaffner¹³⁶. Dowex 1X2(OH form) was the ion-exchange resin and water the eluting solvent. The column eluate was monitored by reaction with ninhydrin. Using a slower elution rate and a smaller particle size of the same ion-exchange resin Inouye and Ogawa¹⁹¹ reported improved resolution of the peaks. The application of this procedure to the quantitative determination of neomycin C and neamine in commercial samples of raw material has also been described⁶. When compared with TLC results on the same samples, the column chromatographic method gave consistently higher results for the neamine content. TLC examination of the

fraction of eluate containing neamine, demonstrated the presence of three other unidentified compounds which gave a positive ninhydrin reaction thus explaining the observed difference between the two procedures.

Roets and Vanderhaeghe¹⁹³ have also examined neomycin by chromatography on Dowex 1X2 but these authors monitored the column eluate conductometrically. Gillet et al¹⁹⁴ applied a similar procedure to the examination of various samples of neomycin to ascertain the ratio of neomycin B:C.

A number of minor components present in commercial neomycin have been separated by column chromatography on a carboxylic cation-exchange resin, Amberlite CG-50¹⁹⁹. The components were eluted from the resin with ammonium hydroxide solution. T.L.C. of the eluent fractions showed the presence of two previously unreported impurities which were then isolated on Dowex 1X2 and tentatively identified using NMR and mass spectrometry.

When investigating the acid hydrolysis of neomycin, Brazhnikova and Kudinova¹⁹⁸ added a sulphocationic-type ion-exchange resin to the acid solution of antibiotic to adsorb intermediate hydrolysis products and thus prevent further decomposition during heating. Following elution of the resin in a column the products of neomycin hydrolysis were identified and shown to be dependant on the time of hydrolysis.

An ion-exchange chromatographic procedure utilising Dowex 1X2 has been made the basis of an industrial process for manufacturing neomycin B which is free from neomycin C¹⁹⁵.

The isolation of neamine on Amberlite IRC 50(Na form) following the acid hydrolysis of neomycin has been described¹⁹⁶. Neamine is eluted from the ion-exchange column with hydrochloric acid. This process had been patented¹⁹⁷ as a means of manufacturing neamine.

Many column chromatographic procedures have been described for the commercial separation and purification of neomycin following fermentation.

6.34. Paper and Thin-Layer Chromatography

The examination of neomycin by paper and thin-layer chromatography has been for two main purposes:-

- a) to separate and identify neomycin in the presence of other antibiotics, and
- b) to separate individual components and degradation products of neomycin.

The solvent systems employed for these purposes are summarised in Tables 12 and 13 (paper chromatography) and Tables 14 and 15 (thin-layer procedures).

Visualisation of the chromatograms has been reported using both chemical and microbiological means. Chemical spray reagents which have been described are listed in Table 16. The various organisms that have been employed for bioautography are given in Table 11.

Table 11
Visualisation of Chromatograms
Bioautographically

<u>Organism</u>	<u>Reference</u>
<i>B. subtilis</i>	201, 202, 204, 228 234, 235
<i>B. pumilus</i>	224
<i>Escherichia coli</i>	205, 206
<i>Staphalococcus aureus</i> ATCC 6538 strain	207, 220, 231, 232

Table 12
Paper Chromatographic Systems Separating Neomycin
from other Antibiotics

<u>Paper</u>	<u>Direction</u>	<u>Solvent System</u>	<u>Rf Value</u>	<u>Use</u>	<u>Reference</u>
Whatman No.1	Ascending	Water-saturated butanol	0.84)	202
Whatman No.1	Ascending	n-Butanol/acetic acid/water(2:1:1)	0.85)	202
Whatman No.1	Ascending	n-Butanol/pyridine/ water(1:0.6:1)	0.94) Identifi-) cation of) neomycin in	202
Whatman No.1	Ascending	3% Aqueous ammonium chloride	0.00) the presence) of other	202
Whatman No.1	Ascending	Benzene/acetic acid/ water(2:1:1), organic layer	0.89) antibiotics))	202
Whatman No.1	Ascending	n-Butanol-saturated water	0.07)	202
	Ascending	Methanol/3% aqueous sodium chloride(5:1)	0.03) Separation of) neomycin from	203
Toyo No.50	Descending for 9 hrs.	n-Butanol/pyridine/ acetic acid/water (15:10:3:12)	0.13) other) streptomyces) antibiotics	204

Table 12(Contd..)

Paper Chromatographic Systems Separating Neomycin
from other Antibiotics

<u>Paper</u>	<u>Direction</u>	<u>Solvent System</u>	<u>Rf Value</u>	<u>Use</u>	<u>Reference</u>
Toyo No.50	Descending for 9 hrs.	2% p-toluene sulphon- ic acid + 2% pyridine in wet n-butanol	0.48-0.54)	Separation of)neomycin from)other)streptomyces)antibiotics.	204
Toyo No.50	Ascending	t-Butanol/acetic acid/ water(55:6:39)	0.06))	204
Whatman No.1	Ascending	Water	0.98))	205
Whatman No.1	Ascending	Water-saturated n-butanol	0.0))Systematic)identification)of the	205
Whatman No.1	Ascending	Water-saturated ethyl acetate	0.0))antibiotics	205
Whatman No.1	Ascending	Water-saturated benzene	0.0))	205
Whatman No.1	Ascending	Methanol/water(40:60)	0.21))	205,206
Whatman No.1	Ascending	n-Propanol/water(40:60)	0.20))	205,206
Whatman No.1	Ascending	Methanol/3% aqueous ammonium chloride(70:30)	0.11))	205,206
Whatman No.1	Ascending	Methyl ethyl ketone/n- butanol/water(30:5:65)	0.27))	205,206

Table 12 (Contd....)

Paper Chromatographic Systems Separating Neomycin
from other Antibiotics

<u>Paper</u>	<u>Direction</u>	<u>Solvent System</u>	<u>Rf Value</u>	<u>Use</u>	<u>Reference</u>
Whatman No.1	Ascending	66.6% aqueous propanol/benzene/ethylene glycol/acetic acid(5:5:1.5:1)	0.03)		207
Whatman No.1	Ascending	50% aqueous propanol/acetic acid(25:1)	0.27)	Separation of neomycin from	207
Whatman No.1	Ascending	Water-saturated butanol/acetic acid/potassium cyanide (100:1:0.05g)	0.00)	chloramphenicol, tetracycline and penicillin	207
Whatman No.3	Ascending	n-Butanol/water/acetic acid(30:13:8)	0.02)	Separation of neomycin from	186,208
Whatman No.3	Ascending	n-Butanol/acetic acid/pyridine/water/ethanol (60:15:6:5:5)	0.04)	polymixin B and bacitracin	186,208
Whatman No.3	Ascending	n-Butanol/water/acetic acid/pyridine/sodium chloride (30:12:7:2:0.1)	0.05)		186,208

Table 13

Paper Chromatographic Systems Separating Neomycin
Components and Degradation Products

<u>Paper</u>	<u>Direction</u>	<u>Solvent System</u>	<u>Rf Value</u>		<u>Use</u>	<u>Reference</u>
			<u>Neomycin</u> <u>B</u>	<u>Neamine</u> <u>C</u>		
-	-	Water/butanol/ methanol/methyl orange (2:4:1:1:5)	Not available		Stability testing fradio- mycin	209
Whatman No.1	Descending	Butanol/pyridine/ water(60:30:40)	0.29	0.19	Separation of B and C as acetyl derivatives	210
-	-	Butanol/pyridine/ water(3:2:1)	Not available		Separation of B and C as acetyl derivatives	211
Toyo No.50	Descending for 9 hrs	n-Propanol/ pyridine/acetic acid/water (15:10:3:12)	0.13 - 0.15	0.28)))))	Separation of neamine from neo- mycins B and C	204

Table 13 (Contd..)

Paper Chromatographic Systems Separating Neomycin
Components and Degradation Products

<u>Paper</u>	<u>Direction</u>	<u>Solvent System</u>	<u>Rf Value</u>			<u>Use</u>	<u>Reference</u>
			Neomycin <u>B</u>	<u>C</u>	Neamine		
Toyo No.50	Descending for 9 hrs	2% p-toluene sulph- onic acid in water saturated n-butanol	0.11-	0.20	0.23) Separation) of neamine) from neo-) mycins B) and C)	204
Toyo No.50	Ascending	1.5% sodium chlor- ide in 80% methanol	0.08-	0.11	0.22)	
Grade M	Ascending	Butanol/acetic acid/water (4:1:5)	Not available			Separation of B and C as acetyl derivatives	212
Whatman No.4	Descending for 24-36 hrs , 28°C	n-Butanol/water/ piperidine (84:16:2)	0.70	0.35	1.00	Quantitative spectrophoto- metric deter- mination of components	213

Table 13(Contd...)

Paper Chromatographic Systems Separating Neomycin
Components and Degradation Products

<u>Paper</u>	<u>Direction</u>	<u>Solvent System</u>	<u>Rf Value</u>			<u>Use</u>	<u>Reference</u>
			Neomycin B	Neamine C			
Whatman No.1	Descending for 24-36 hrs , 25- 27°C	Methyl ethyl ketone/t-butanol/ methanol/6.5N ammonium hydroxide (16:3:1:6)	0.54 (values relative to neamine)	0.30	1.00	Separation as <u>free bases</u> Quantitation by spectro- photometry	214
Whatman No.1	Descending for 8-40 hrs , 24°C	Methyl ethyl ketone/ isopropanol/6.5N ammonium hydroxide (80:20:30)	0.57 (values relative to neamine)	0.34	1.00		215
-	Radial	Butanol/pyridine/ acetic acid/water (15:10:3:12)	Not available			Separation of degradation products	216
Whatman No.1 im- pregnat- ed with pH 8 buffer	Descending for 18 hrs	Butanol/water(1:2) containing 4% toluenesulphonic acid and 2% piperidine hydrochloride	0.5	0.6	0	Separation of neamine from neomycin B and C	228

Table 14

Thin Layer Chromatographic Systems Separating
Neomycin from Other Antibiotics

<u>Adsorbent</u>	<u>Solvent System</u>	<u>Rf Value</u>	<u>Use</u>	<u>Reference</u>
Silica gel G/ aluminium oxide (1:1)	n-Propanol/ethylacetate/ water/13.4 M ammonium hydroxide (50:10:30:10)	0.52	Separation of neomycin from other aminoglyco- side antibiotics	217
Cellulose MN	n-Propanol/pyridine/ acetic acid/water (15:10:3:12)	0.10	Separation of neomycin from other water solu- ble basic antibiotics	218
Kieselgel G	Water/methanol/butanol/ butyl acetate/acetic acid(12:2.5:7.5:40:20)	0.0)) Separation of) neomycin from) bacitracin,) polymixin and) tyrothricin	219
Kieselgel G	Water/butanol/pyridine/ acetic acid. (14:30:20:6) (16:40:8:16)	0.12) 0.00))		219
Kieselgel G	Water/butanol/acetic acid(39:55:6)	0.00)		219

Table 14 (Contd...)

Thin Layer Chromatographic Systems Separating Neomycin
from Other Antibiotics

<u>Adsorbent</u>	<u>Solvent System</u>	<u>Rf Value</u>	<u>Use</u>	<u>Reference</u>
Kieselgel G	Methanol/17% aqueous ammonia chloroform, aqueous layer (10:10:20)	0.31) Separation of neomycin from bacitracin, polymixin and tyrothricin	219
Sephadex G-15	pH6 phosphate buffer (0.025M)+ 0.5M sodium chloride	0.75) Identification of antibiotics	220
Silica gel G	Benzene/acetone/acetic acid (4:4:2)	0.35)	221
Kieselgel G	Butanol/acetic acid/pyridine/water (30:22:6:38)	0.14) Separation of neomycin from zinc bacitracin and polymixin	186,208
Kieselgel G	Butanol/water/acetic acid/ethanol/pyridine (60:10:15:5:6)	0.05)	186,208

Table 14 (Contd...)

Thin Layer Chromatographic Systems Separating
Neomycin from Other Antibiotics

<u>Adsorbent</u>	<u>Solvent System</u>	<u>Rf Value</u>	<u>Use</u>	<u>Reference</u>
Kieselgel G	Propanol/ethyl acetate/water/ 25% ammonium hydroxide	0.08)		222
Kieselgel G + Kieselguhr G(1:1)	(100:20:60:20).	0.14)		222
Kieselgel G + Kieselguhr G(1:2)	"	0.22)		222
Cellulose MN-300	"	0.32)	Separation of glycosidic antibiotics	222
Kieselgel G + aluminium oxide G type E(1:1)	"	0.12)		222
Kieselgel G impregnated with sodium acetate	"	0.33)		222

Table 14 (Contd..)

Thin Layer Chromatographic Systems Separating
Neomycin from Other Antibiotics

<u>Adsorbent</u>	<u>Solvent System</u>	<u>Rf Value</u>	<u>Use</u>	<u>Reference</u>
Kieselgel G	Propanol/ethyl acetate/ water/25% ammonium hydroxide/	0.11)		222
Kieselgel G	pyridine/3.85% aqueous	0.13)		222
+ Kieselguhr	ammonium acetate)		
G(1:1)	(100:20:60:20:10:200))		
Kieselgel G	"	0.16)		222
+ Kieselguhr)		
G(1:2))		
Kieselgel G	"	0.08)		222
impregnated)		
with 3.85%)	Separation of glycosidic antibiotics	
aqueous)		
ammonium)		
acetate)		
Kieselgel G	Ethanol/ethylacetate/water/ 25% ammonium hydroxide/	0.08)		222
Kieselgel G	pyridine/3.85% aqueous	0.11)		222
+ Kieselguhr	ammonium acetate)		
G(1:1)	(100:20:60:20:10:200))		
Kieselgel G	"	0.12)		222
+ Kieselguhr)		
G(1:2))		

Table 14 (Contd..)

Thin Layer Chromatographic Systems Separating
Neomycin from Other Antibiotics

<u>Adsorbent</u>	<u>Solvent System</u>	<u>Rf Value</u>	<u>Use</u>	<u>Reference</u>
Kieselgel G + aluminium oxide G (type E) (1:1)	Ethanol/ethylacetate/water/ 25% ammonium hydroxide/ pyridine/3.85% aqueous ammonium acetate (100:20:60:20:10:200)	0.08))))))		222
Kieselgel G	Methanol/ethyl acetate/ water/25% ammonium hydrox-	0.06))		222
Kieselgel G + Kieselguhr G(1:1)	ide/pyridine/3.85% aqueous ammonium acetate (100:20:60:20:10:200)	0.17)))	Separation of glycosidic antibiotics	222
Kieselgel G + Kieselguhr G(1:2)	"	0.14)))		222
Kieselgel G + aluminium oxide G, type E(1:1)	Methanol/ethyl acetate/ water/25% ammonium hydrox- ide/pyridine/3.85% aqueous ammonium acetate (100:20:60:20:10:200)	0.11)))))		222

Table 14 (Contd..)

Thin Layer Chromatographic Systems Separating
Neomycin from Other Antibiotics

<u>Adsorbent</u>	<u>Solvent System</u>	<u>Rf Value</u>	<u>Use</u>	<u>Reference</u>
Kieselgel G impregnated with 3.85% aqueous ammonium acetate	Methanol/ethyl acetate/ water/25% ammonium hydrox- ide/pyridine/3.85% aqueous ammonium acetate (100:20:60:20:10:200)	0.08))))))))		222
454 Kieselgel G	Butanol/methanol/ethyl acetate/water/25% ammonium	0.06))		222
Kieselgel G + Kieselguhr G(1:1)	hydroxide/pyridine/3.85% aqueous ammonium acetate (100:100:20:60:20:10:200)	0.07))		222
Kieselgel G + Kieselguhr G(1:2)	"	0.13))) Separation of) glycosidic) antibiotics	222
Kieselgel G + aluminium oxide G, type E(1:1)	"	0.05))))		222
Kieselgel G + Kieselguhr G(1:2)	25% ammonium hydroxide/ water/acetone (16:144:40)	0.36)))		222

Table 14 (Contd..)

Thin Layer Chromatographic Systems Separating
Neomycin from Other Antibiotics

<u>Adsorbent</u>	<u>Solvent System</u>	<u>Rf Value</u>	<u>Use</u>	<u>Reference</u>
Kieselgel G + Kieselguhr G(1:2) impreg- nated with 2% sodium acetate	25% ammonium hydroxide/ water/acetone (16:144:40)	0.34))))))		222
Kieselgel G + aluminium oxide G, type E(1:1)	"	0.48)))))	Separation of glycosidic antibiotics	222
Kieselgel G + Kieselguhr G(1:1) impreg- nated with 2% sodium acetate	"	0.43)))))		222
Kieselgel G	Water/sodium citrate/ citric acid (100:20:5)	0.95	Classification of antibiotics	223

Table 15

Thin Layer Chromatographic Systems Separating
Neomycin Components and Degradation Products

<u>Adsorbent</u>	<u>Solvent System</u>	<u>Rf Value</u>			<u>Use</u>	<u>Reference</u>
		<u>Neomycin B</u>	<u>Neomycin C</u>	<u>Neamine</u>		
Acidified carbon	Water	0.10	0.10	0.60) Assay of) neomycin) components	224
Acidified carbon	0.5N sulphuric acid	0.21	0.43	0.61) in commer-) cial form-) ulations	224
Untreated carbon	Water	0.00	0.00	0.00)))	224
Untreated carbon	0.5N sulphuric acid	0.24	0.45	0.54)	224
Carbon treated with hydrochloric acid	0.5N hydrochloric acid	0.37-0.45	0.53-0.74	0.63-0.92))) Separation) of neomycins) B and C and) neamine	204
"	0.5N hydrochloric acid/ methanol(4:1)	0.44-0.72	0.58-0.81	0.64-0.91)))	204

Thin Layer Chromatographic Systems Separating Neomycin Components and Degradation Products

<u>Adsorbent</u>	<u>Solvent System</u>	<u>Rf Value</u>			<u>Use</u>	<u>Reference</u>
		<u>Neomycin B</u>	<u>Neomycin C</u>	<u>Neamine</u>		
Carbon treated with water	0.5N hydro- chloric acid	0.41	0.57	0.62)	204
"	0.5N hydro- chloric acid/ methanol(4:1)	0.48	0.58	0.68)	204
Carbon/ gypsum treated with sul- phuric acid	0.5N sulphuric acid	0.20	0.59	0.80) Separation) of neomycins) B and C and) neamine	204
"	0.5N sulphuric acid/methanol(4:1)	0.64	0.82	0.85)	204
Carbon/ gypsum treated with water	0.5N hydro- chloric acid	0.16	0.28	0.30)	204

Table 15 (Contd..)

Thin Layer Chromatographic Systems Separating
Neomycin Components and Degradation Products

<u>Adsorbent</u>	<u>Solvent System</u>	<u>Rf Value</u>			<u>Use</u>	<u>Reference</u>
		<u>Neomycin B</u>	<u>Neomycin C</u>	<u>Neamine</u>		
Carbon/ gypsum treated with water	0.5N sulphuric acid	0.24	0.40	-))))) Separation	204
"	0.5N hydro- chloric acid/ methanol (1:1)	0.36	0.36	0.46) of neomycin) B and C and) neamine)	204
"	0.5N hydro- chloric acid/ propanol (1:1)	0.48	0.48	0.52)))	204
Kieselgel H	3.85% aqueous ammonium acetate	0.13	0.13	0.36	Assay of neamine	196,9
Kieselgel H	3.4% ammonium hydroxide	0.37	0.44	0.47	Assay of neomycin C	196,9

Table 15 (Contd..)

Thin Layer Chromatographic Systems Separating
Neomycin Components and Degradation Products

	<u>Adsorbent</u>	<u>Solvent System</u>	<u>Rf Value</u>			<u>Use</u>	<u>Reference</u>
			<u>Neomycin B</u>	<u>Neomycin C</u>	<u>Neamine</u>		
	Cellulose MN	Methyl ethyl ketone/iso- propanol/6.5N ammonium hydr- oxide (80:20:30)	0.21	0.12	0.31	Separation of neomycin components	215
459	Cellulose MN-300	Methyl ethyl ketone/methanol/ isopropanol/7.9N ammonium hydr- oxide (10:8.5:3:7)	0.52	0.24	0.61		236
	Cellulose MN-300	Propanol/pyridine/ acetic acid/water (100:66:20:80)	0.25	0.20	0.47	single development) Assay of double development) neomycins B 0.33 0.25 0.60) and C and nea- mine in pharm- aceutical form- ulations	226
	Silica gel G	"	0.57	0.66	0.73		226

Table 15 (Contd..)

Thin Layer Chromatographic Systems Separating
Neomycin Components and Degradation Products

	<u>Adsorbent</u>	<u>Solvent System</u>	<u>Rf Value</u>			<u>Use</u>	<u>Reference</u>
			<u>Neomycin B</u>	<u>Neomycin C</u>	<u>Neamine</u>		
460	Cellulose MN-300/ Kieselguhr G(1:1)	Propanol/pyridine/ acetic acid/water (100:66:20:80)	0.53	0.46	0.73)))))		226
	Silica gel G	Propanol/ethyl acetate/water/25% ammonium hydroxide (100:20:60:20)	0.23	0.23	0.42)))) Assay of) neomycins B		226
	Cellulose MN-300/ Kieselguhr G(1:1)	Methanol/3% aqueous sodium chloride (2:1)	0.36	0.17	0.43)) and C and) neamine in) pharmaceutical) formulations)		226
	Silica gel G/ Kieselguhr G(1:2)	3.85% aqueous ammonium acetate	0.22	0.22	0.39))))		226

Table 15 (Contd..)

Thin Layer Chromatographic Systems Separating
Neomycin Components and Degradation Products

<u>Adsorbent</u>	<u>Solvent System</u>	<u>Rf Value</u>			<u>Use</u>	<u>Reference</u>
		<u>Neomycin B</u>	<u>Neomycin C</u>	<u>Neamine</u>		
Silica gel G	Methyl ethyl ketone/t- butanol/methanol/ 6.5N ammonium hydroxide (160:30:10:60)	0.14	0.11	0.22)	226
)	
)	
)	
)	
)	
Kieselguhr G purified	"	0.46	0.68	0.84) Assay of neomycins B and C and neamine in pharmaceutical formulations	226
Silica gel G	Propanol/ethan- ol/ethyl acetate/ water/25% ammonium hydroxide/pyridine/ 3.85% ammonium acetate (100:100:20:60:20: 10:200)	0.18	0.15	0.38)	
)	
)	
)	
Silica gel G/ Kieselguhr G(1:2)	"	0.18	0.11	0.32)	226

Table 15 (Contd..)

Thin Layer Chromatographic Systems Separating
Neomycin Components and Degradation Products

<u>Adsorbent</u>	<u>Solvent System</u>	<u>Rf Value</u>			<u>Use</u>	<u>Reference</u>
		<u>Neomycin B</u>	<u>Neomycin C</u>	<u>Neamine</u>		
Dowex 50X8 sodium form(Ionex 25SA)	1.5M Sodium acetate, pH 8.5 + 58.4g sodium chloride/ t-butanol(10:1)	0.15	0.15	0.28	Separation of neamine from neomycins B and C	227
Silica gel	3% Ammonium hydroxide/acetone (160:40)	0.33	0.33	-	Quantitative analysis of neomycin sulphate	229
Kieselgel G	3% Ammonium hydroxide/acetone (160:90)	Not available			Stability of neomycin after ethylene oxide sterilisation	233

Table 16
Visualisation of Chromatograms by
Chemical Means

<u>Spray Reagent</u>	<u>Colour of Neomycin zone</u>	<u>Reference</u>
1. Ethanolic sodium hypochlorite followed by starch/potassium iodide solution	Dark blue	210,211
2. Chlorine/ethanol followed by starch/potassium iodide solution	Dark blue	212
3. Chlorine/carbon tetrachloride followed by starch/potassium iodide/pyridine	Dark blue	213
4. t-butyl hypochlorite/dichloromethane/acetic acid followed by starch/potassium iodide solution	Dark blue	196
5. Ninhydrin	Purple	186,203,208 214,215,216 217,218,219 229,231
6. Ninhydrin/cadmium acetate	Purple/pink	227
7. p-dimethyl amino-benzaldehyde	Pink or purple	223
8. Ninhydrin/p-dimethyl amino-benzaldehyde		226
9. Copper sulphate/ammonium hydroxide	Blue	221

Quantitative analysis of neomycin after chromatography utilising some of the visualisation techniques given in Tables 11 and 16 have been reported. Majumder and Majumder²¹³ separated neomycins B and C as the acetyl derivatives on paper then, following conversion to the chloro derivatives, the colour formed by reaction with the starch/iodine/hydrochloric acid reagent was measured at 570nm. A later paper by the same authors²¹⁴ described the separation of neomycins B and C as the free bases. The resulting chromatograms were developed with ninhydrin then the coloured complex eluted into methanol and the absorbance of the solution measured at 570nm.

Thin-layer chromatography has been used by Foppiano and Brown²²⁹ to assay the total neomycin B and C content of neomycin sulphate. The neomycin zone was scraped off the plate and reacted with orcinol/ferric chloride reagent, the absorbance of the resulting colour being measured at 665nm. By this procedure a precision of $\pm 2\%$ was achieved.

Ninhydrin has also been used to quantitate neomycin colorimetrically following thin-layer chromatography. Pregnotatto and Sabino²⁰⁰ reported the addition of glycerine to the ninhydrin solution to improve the sensitivity and reproducibility of the procedure. An alternative colorimetric procedure for the quantitation of neomycin after chromatography has been described by Doullakas¹⁷⁴. Neomycin is eluted from the silica gel with pH 12.5 phosphate buffer then oxidised with sodium hypobromite. The resulting aldehyde is complexed with phloroglucinol yielding a pink-coloured product which is measured spectrophotometrically.

The use of bioautography for the quantitation of chromatograms has been described by many workers. Emilianowicz-Czerska and Herman²²⁸ separated neamine from neomycins B and C then quantitatively determined total neomycin B and C by bioautography with *B. subtilis*. A linear response for concentrations between 0.25 and 10 μ g of neomycin was obtained. With a similar technique but using a mathematical analysis of the bioauto-

grams, Simon²³⁰ reported a quantitative determination of neomycin suitable for use with irregular-shaped zones. Brodasky²²⁴ employed bioautography with *Bacillus pumilus* for the assay of neomycin and neamine after separation by T.L.C. on a layer of carbon. The quantitation of small amounts of neomycin C however, proved difficult, the difficulties being associated with the enhanced growth of the organism in the presence of carbon (from the T.L.C. plate) and ammonium ions (from the chromatographic procedure). Similar difficulties were experienced by Sokolski and Lummis²³⁴ who attributed poor neomycin zones to be the result of antagonism by the sodium chloride present in the bioautographic system. An extensive study by Langner²⁰¹ demonstrated maximum sensitivity for the determination of neomycin to be apparent using the organism *B. subtilis* (ATCC 6633) together with pH 8 Tris agar. The detection limit following chromatography was reported to be 0.05µg.

6.35. Gas-Liquid Chromatography

Tsuji and Robertson¹⁹² achieved the separation of neomycin B, neomycin C and neamine as the trimethylsilyl ethers on a 6ft. column of 0.75% OV-1 on Gas Chrom Q at a temperature of 290°C. The same conditions have also been shown to separate neobiosamine B, neosamine and deoxystreptamine from neomycin and neamine. Hence the method could be used to study the stability of neomycin or to monitor the biosynthetic production process. Use of the procedure to assess the stability of neomycin in pharmaceutical formulations has been demonstrated by Van Giessen and Tsuji²³⁷ with trilaurin as internal standard. However, these authors recommended a 2ft. column packed with 3% OV-1 on Gas Chrom Q as longer columns required a higher temperature to chromatograph neomycin and consequently had a reduced column life. A concentration of 3% OV-1 was preferred, as 2% or less resulted in increased column adsorption of neomycin. After a further study of the procedure Margosis and Tsuji²³⁸ recommended a number of improvements to optimise the analysis of neomycin by G.L.C. The improvements to the assay included the modification of the injection port to prevent sample decomposition by contact of injected material with

metal or Teflon and the addition of an accurate volume of silylating reagent to each sample and standard to overcome inconsistent derivatisation.

A comparison of the G.L.C. and microbiological (agar-diffusion) assays has been reported by Tsuji et al²³⁹ who showed the neomycin content of neomycin sulphate powders determined by the G.L.C. method, to correlate well with values obtained by the microbiological assay when it is assumed neomycin C has 35% of the biological activity of neomycin B.

Neomycin has been separated from mixtures of other aminoglycoside antibiotics containing the 2-deoxystreptamine moiety as both the pertrimethylsilyl derivative and the N-trifluoroacetyl pertrimethylsilyl derivative using a column of 0.75% OV-1 on Gas Chrom Q²⁴⁰. The procedure may be used to estimate the number of sugar moieties bound in the antibiotic as a close relationship exists between the number of rings and the retention time.

Examination of neomycin B by a combined G.C.-M.S. procedure has been reported by Murata et al¹⁵. G.C. was accomplished with the trimethylsilyl derivative on a 1m column of 1% OV-1 on Chromosorb W, by which means neomycin was separated from kanamycin. The resulting mass spectra of the neomycin derivative exhibited a minute molecular ion peak at m/e 1550 indicating that all active hydrogens of both hydroxy and amine groups were completely silylated.

6.4. Microbiological Procedures

6.41. Turbidimetric Assay

Various organisms have been used to assay neomycin turbidimetrically. These are summarised in Table 17 with the working concentration for the antibiotic as reported by the respective authors.

Of the organisms tabulated overleaf *K.pneumoniae* is generally used for routine assays.

Table 17
Conditions of Turbidimetric Assay

<u>Organism</u>	<u>Concentration Range of Assay</u>	<u>Reference</u>
	(Total µg neomycin base required in solution to be assayed)	
<i>S. aureus</i> NRRLB314	2.5 - 5.0	241
" " 209P	100-300	242
" " F1	0.016 - 0.04	243
<i>Streptococcus</i> <i>faecalis</i> ATCC 1054	120-200	248
<i>Klebsiella</i> <i>pneumoniae</i> PC1602	6-15	244
<i>Klebsiella</i> <i>pneumoniae</i> ATCC 10031	6-14	245, 246, 268
<i>E. coli</i> ATCC 10536	1-3	247
" " ATCC 11105	1-20	253
<i>Lactobacillus</i> <i>arabinoxus</i>	3-7	248
<i>Lactobacillus</i> <i>casei</i>	20-60	248
<i>Lactobacillus</i> <i>fermenti</i>	2-6	248
<i>Lactobacillus</i> <i>leichmanni</i>	4-6	248
<i>Aerobacter</i> <i>aerogenes</i> IPL 22K5118	0-6	249

Hein²⁴³ noted an apparent decrease in the potency of neomycin when assayed turbidimetrically if the inoculum contained either sodium chloride or potassium phosphate. With *S. aureus* as organism, Sokolski et al²⁵² demonstrated the presence of KCl to antagonise the activity of neomycin C more than that of neomycin B.

A comparative study of the response of neomycin B and neomycin C in the turbidimetric assay using *K.pneumoniae*(ATCC 10031) has been reported²³⁹. The response-ratio neomycin B: neomycin C was 100:38.9.

An automated turbidimetric procedure using a Technicon Autoanalyzer has also been described (See Section 6.5).

6.42. Agar-Diffusion Assay

Table 18 lists the organisms that have been recommended for determining the microbiological potency of neomycin.

Table 18

Recommended Organism	Working Concentration ($\mu\text{g/ml}$)	Reference
<i>B.pumilis</i> NCTC 8241	4-21	9
<i>S.aureus</i> ATCC 6538	4-20	245
<i>S.epidermidis</i> ATCC 12228	0.64 - 1.56	245,268

In all the above references, the cylinder-plate assay procedure is the one described though alternative procedures have been reported in the literature. Thus Davis and Parke²⁴⁵ reported a linear-diffusion system in which a solution of the antibiotic is allowed to diffuse into agar-filled glass capillaries. The standard deviations calculated for seven experiments were between 5 and 10%(100 results) for the assay of neomycin. The procedure is more economic in terms of agar and nutrients than other diffusion methods. Kuzel and Coffey²⁵⁵ modified the conventional cylinder plate procedure by using a cylinder sealed at one end, forming a cup. The cups are filled with the antibiotic solutions to be assayed, then a plate of inoculated agar is inverted and placed over the top of the cups. With this method a significant reduction in the standard deviation for the assay of neomycin

was claimed. The replacement of cylinders by paper discs has also been reported^{256,275,278}. Rossi²⁷⁵ claimed a standard deviation of $\pm 2\%$ for the assay of neomycin by this means and recommended the paper disc procedure in preference to the cylinder method. The type of paper used has been shown to affect the diameter of the inhibition-zone²⁷⁶ Whatman seed test paper producing the smallest zones. When determining neomycin levels in milk, Kosikowski²⁷⁷ improved the sensitivity of the procedure by substituting dried milk tablets for paper discs.

Several studies of the factors affecting the inhibition-zone size in the neomycin agar diffusion assay have been reported. Pinzelik et al²⁵⁷ demonstrated that various delays during the analytical procedure affected the size of the inhibition zone. Refrigeration of the agar-plates prior to incubation caused an increase in zone-size when compared with similar plates held at 20°C. This principle has been utilized by Siddique et al²⁶⁴ to improve sensitivity when determining the neomycin content of milk. The presence of KCl, NaCl, CaCl₂ has been shown to markedly increase neomycin diffusion in agar^{258,265,194}. Conversely the presence of phosphates²⁵⁸ and of glucose/starch with NaCl²⁶⁵ decreases the size of the inhibition zone. Incorporation of non-fat milk in the agar significantly reduces the size of the inhibition zone because of the interaction of neomycin with the free carboxyl group of milk protein²⁶². Fedorko and Katz²⁶³ diluted neomycin solutions with blood serum and reported an increase in the size of the inhibition zone when compared with similar solutions diluted with pH 8 phosphate buffer. Sokolski et al²⁵² and Yousef et al²⁵⁹ demonstrated the physical binding of neomycin to agar, a process which may be reversed by addition of potassium or sodium chloride. Further studies^{260,261,252} have reported the binding to be a function of both the pH of the agar and the manufacturer. Incorporation of 0.1M tris buffer with the agar (pH 8) has been recommended^{260,261} in order to achieve the highest sensitivity. Tris buffer results in a greater sensitivity than phosphate buffer²⁶¹ and does not affect the size of the inhibition zone whereas the presence of phosphate reduces zone size²⁵². Gillet et al¹⁹⁴ concluded that the quality of the agar affect-

ted the inhibition zone size. A comparison of 3 agars showed the best response for a given concentration of neomycin was obtained with agarose. Combining agarose with recommendations described by other authors, such as the addition of CaCl_2 and the use of tris buffer, further increased the size of the inhibition zone.

The literature described above refers to the assay of the total neomycin complex i.e. neomycins B and C and neamine. To assay neomycin B in the presence of neomycin C and neamine by a microbial method Sokolski and Carpenter²⁶⁵ adopted the following procedure. These authors employed a neomycin C - resistant organism (*B.subtilis* UC 564) and added KCl to the agar to totally depress diffusion of neamine and increase diffusion of neomycin B.

Neomycin B and C differ in their biological activities though this difference may vary with the conditions of the assay. Typically neomycin C has an activity of 30-50% of that of neomycin B^{266,239}. The presence of potassium chloride or phosphate reduces the diffusion of neomycin C more than that of neomycin B²⁵². Furthermore, this antagonistic effect of the salts has been shown to be more pronounced when using *S.aureus* as organism than when using *B.subtilis*. By altering the ionic strength of the medium a system has been developed in which the responses of neomycins B and C are identical²⁶⁶.

The microbial assay of neomycin in the presence of other antibiotics can present difficulties if both antibiotics are active against the same test organisms. The assay of neomycin in the presence of dihydrostreptomycin, to which the test organism *S.aureus* is also sensitive, has been reported. Levine et al²⁶⁹ rendered the dihydrostreptomycin inactive by hydrolysis with barium hydroxide prior to determining neomycin with *S.aureus*. DeNuzio²⁷⁰ however, chose to overcome the same problem by cultivating a strain of *S.aureus* which was resistant to dihydrostreptomycin. When assaying neomycin in a mixture containing tetracycline, penicillin and dihydrostreptomycin, Tanguay et al²⁷¹ found it necessary to

separate tetracycline and penicillin by solvent extraction before determining neomycin by the method of DeNuzio.

The determination of neomycin in animal feeds has received much attention. Barbiers and Neff²⁷² recommended the use of a tris buffer at pH 8 to prepare neomycin solutions and the addition of magnesium or calcium chloride to the agar for enhancement of the inhibition zones. Extraction of neomycin from the feed required the use of sodium chloride solution for 100% recovery. A collaborative study of this procedure by eighteen laboratories resulted in an average recovery of 100.3% with a coefficient of variation of 15.2%²⁷⁹. Modification of the previous method has been described by Williams and Wornick²⁷³ who incorporated the tris buffer in the agar and deleted the addition of calcium chloride. A three-fold increase in sensitivity was reported. However, a collaborative study of the modified procedure resulted in an average recovery of 115% with a coefficient of variation of 20.4%²⁷⁴.

An investigation of the agar diffusion assay of neomycin at very low levels (50ng-1000ng) in meat products has been reported²⁸⁰. The points on the dose-response graph covering the above concentration range were scattered about the average straight line. However, by joining all the points together a complex curve resulted, the equation of which was derived.

6.5. Automated Procedures

An attempt to automate the turbidimetric method for the determination of neomycin with *K.pneumoniae* has been reported by Gerke et al²⁸¹ who obtained satisfactory assays with solutions containing 150-1200 µg/ml of neomycin. An automated respirometric method, which measured the amount of CO₂ produced by interaction of antibiotic and the bacteria *E.coli* was also reported with a similar sensitivity. In both methods Auto Analyzer systems were employed.

The modification of the above respirometric procedure to include continuous culture of

the test organism *E.coli* has been described²⁸². With the organism grown in this manner the sensitivity of the assay is improved. Greely et al²⁸³ have applied the automated respirometric method to the determination of neomycin in pharmaceutical products and compared these assays with the results obtained by the cylinder-plate procedure on the same samples. Good correlation between the two procedures was demonstrated.

An automated colorimetric assay for the quantitation of the separate components of neomycin (B, C and neamine) has also been reported¹⁴⁰. The method utilizes a separation of the components on a column of carbon and Kieslguhr G (4:1). The column eluate is reacted with ninhydrin to determine the amounts of neomycin B, C and neamine.

6.6. Use as an Analytical Reagent

Turbidimetric procedures for determining ribonuclease²⁸⁴ and deoxyribonuclease²⁸⁵ using neomycin as the precipitant have been described. The turbidity was shown to be dependant on the relative concentrations of reactants, the molecular weight of the RNA or DNA and the ionic strength of the solution thus necessitating the careful control of assay conditions. Extraneous proteins do not interfere enabling the procedure to be applied directly to blood serum.

6.7. Determination in Body Fluids and Tissues

An agar diffusion assay, based upon the method described by Groves and Randall²⁴⁶, has been utilised by the majority of workers for determining neomycin levels in clinical samples. To compensate for the protein-binding effect exhibited by neomycin, Kivman and Geitman³⁰⁹ recommended the addition of serum and 3% KCl to the neomycin standard solution. Danielova³¹⁰, however, preferred to release the bound antibiotic by enzymatic hydrolysis prior to microbiological assay. An agar diffusion method requiring only 20 μ l of serum sample has been described by Axline et al³¹¹.

The application of a turbidimetric method to the examination of serum samples has been reported by Felsenfeld³¹².

More recently a laser light scattering bioassay for determining the neomycin content of milk, serum, urine and bile has been reported³¹³. The procedure uses a helium-neon laser light-source and has a linear dose-response graph over the range 0.1 to 10 μ g/ml for serum.

An enzymic assay involving the reaction of neomycin with aminoglycoside 4'adenyltransferase has been described³¹⁴. A linear response for neomycin was observed over the range 2.5 to 20 μ g/ml serum.

The concentration of neomycin in urine has been determined with a fluorimetric method³¹⁵. Interfering amines were separated by chromatography on SE Sephadex C-25 before reacting the isolated neomycin with fluorescamine.

Brammer and Hemson¹⁸² employed an electrophoretic procedure to separate neomycin from blood-proteins before determining the neomycin concentration colorimetrically.

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This profile attempts to cover the published literature on neomycin up to and including Chemical Abstracts, Volume 85.

PSEUDOEPHEDRINE HYDROCHLORIDE

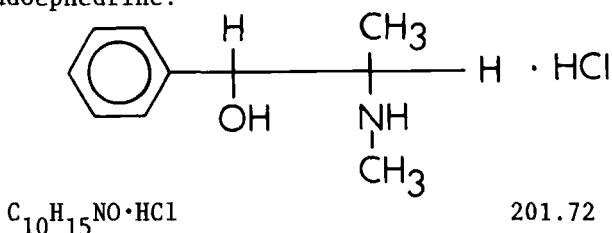
Steven A. Benezra and John W. McRae

1. Description
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1. Description

1.1 Name, Formula, Molecular Weight

d-Pseudoephedrine hydrochloride is (+)-threo- α -(1-(methylamino)ethyl)benzyl alcohol hydrochloride. Throughout this analytical profile, d-pseudoephedrine will be referred to as pseudoephedrine.



1.2 Appearance, Color, Odor

Pseudoephedrine hydrochloride occurs as fine white to off-white crystals or as a powder having a faint odor¹

2. Physical Properties

2.1 Infrared Spectrum

The infrared spectrum of pseudoephedrine hydrochloride is shown in Figure 1. It was obtained as a 0.2% dispersion of pseudoephedrine hydrochloride in KBr with a Nicolet Model 7199 FT-IR spectrophotometer.² Table I gives the infrared assignments consistent with the structure of pseudoephedrine hydrochloride.

Table I
Infrared Spectral Assignments for Pseudoephedrine
Hydrochloride

<u>Frequency (cm⁻¹)</u>	<u>Assignment</u>
3270	OH stretch
3010	Asym. C-H stretch

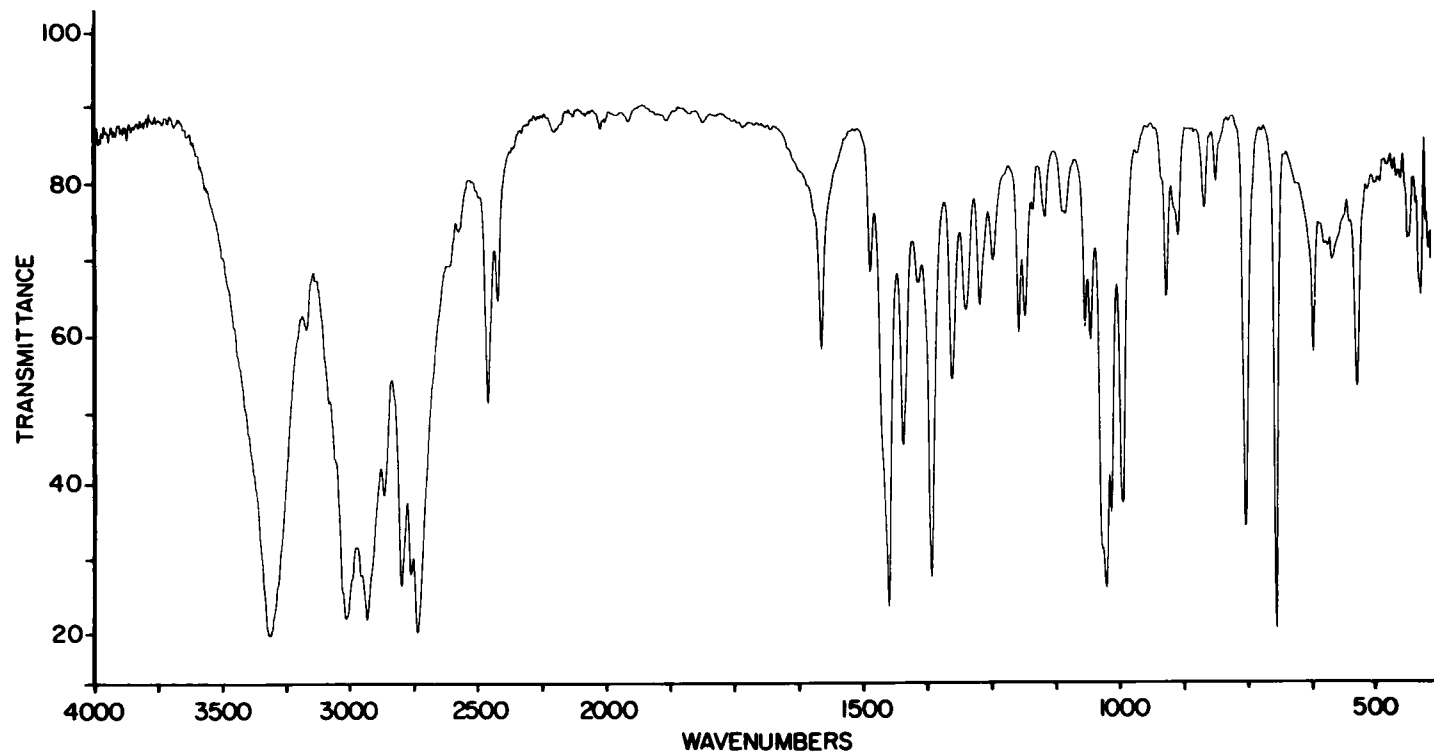


Figure 1 - Infrared Spectrum of Pseudoephedrine Hydrochloride

2930	Sym. C-H stretch
2700	⁺ NH stretch
1587, 1490	C=C aromatic stretch
1430	OH bend, secondary alcohol
762, 702	C-H bend, monosubst. benzene

2.2 Nuclear Magnetic Resonance (NMR) Spectrum

The NMR spectrum of pseudoephedrine hydrochloride is shown in Figure 2. The spectrum was obtained with a Varian model CFT-20 80 MHz NMR spectrometer. Deuterated DMSO was used as the solvent with tetramethylsilane as an internal standard. Table II gives the NMR assignments consistent with the structure of pseudoephedrine hydrochloride.³

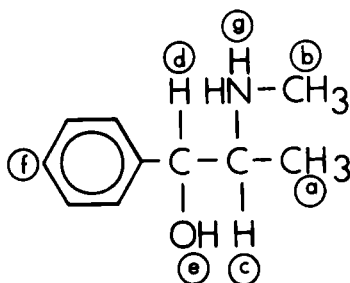


Table II

NMR Assignments for Pseudoephedrine Hydrochloride

<u>Proton</u>	<u>No. of Protons</u>	<u>Shift (ppm)</u>	<u>Multiplicity</u>
a	3	0.96	doublet
b	3	2.55	singlet
c	1	3.25	quartet (partially obscured by H ₂ O)
d	1	4.54	doublet of doublets
e	1	6.32	doublet
f	5	7.34	singlet
g	2	8.90	broad singlet

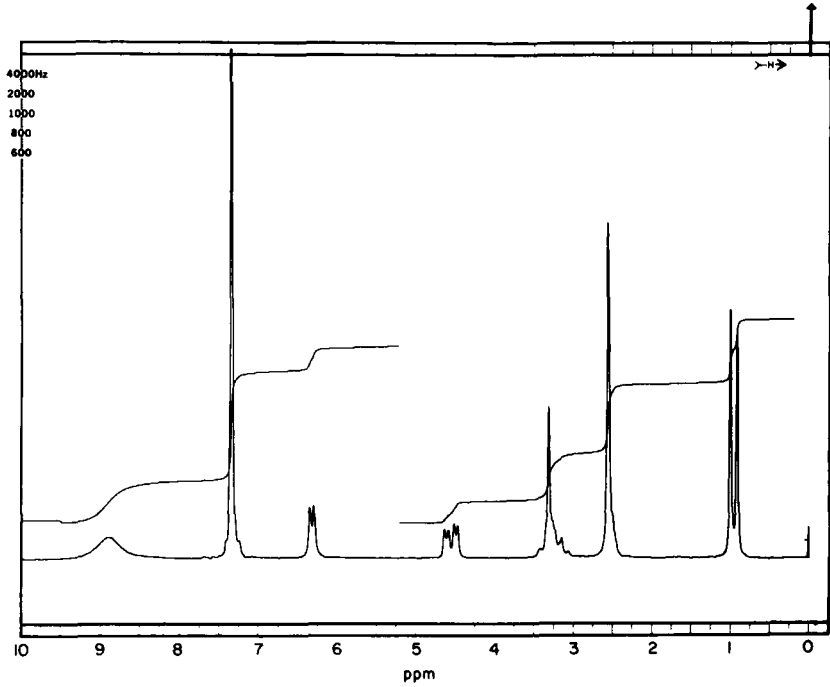


Figure 2 - NMR Spectrum of Pseudoephedrine Hydrochloride

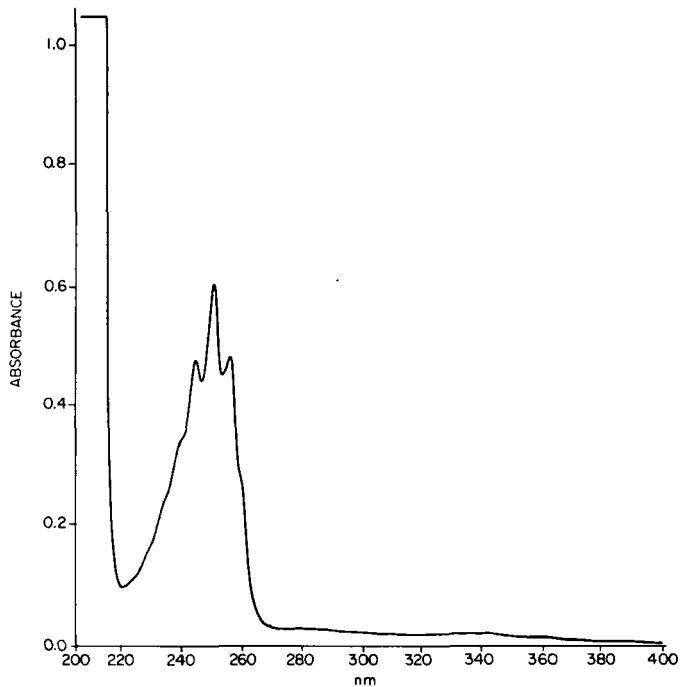


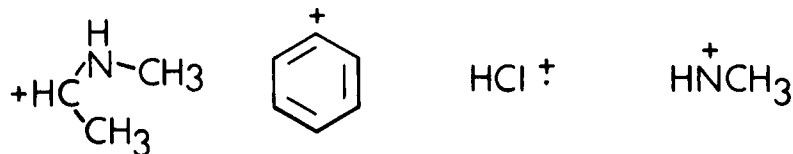
Figure 3 - Ultraviolet Spectrum of Pseudoephedrine Hydrochloride

2.3 Ultraviolet Spectrum

The ultraviolet spectrum of pseudoephedrine hydrochloride in ethanol was obtained with a Beckman ACTA CIII ultraviolet spectrophotometer and is shown in Figure 3.⁴ Pseudoephedrine hydrochloride exhibits absorption maxima at 208, 251, 257, and 264 nm with extinction coefficients of 8300, 161, 201, and 161, respectively.

2.4. Mass Spectrum

The low resolution mass spectrum of pseudoephedrine hydrochloride is shown in Figure 4.⁵ It was obtained with a Varian MAT CH5-DF mass spectrometer. Direct probe at 80°C into the ion source was used to obtain the mass spectrum. The electron energy was 70 eV. The assignments of the major ions formed in the mass spectrometer are shown below. The molecular ion is not observed.



m/e 58 (100%) m/e 77 (9%) m/e 36 (10%) m/e 30 (12%)

2.5 Melting Point

Pseudoephedrine hydrochloride melts between 182° and 185°C.¹

2.5 Specific Rotation

The specific rotation, $[\alpha]_D^{20}$, of d-pseudoephedrine hydrochloride in water is between +61.0° and +62.5°.¹

2.7 Solubility

The solubility of pseudoephedrine hydrochloride in

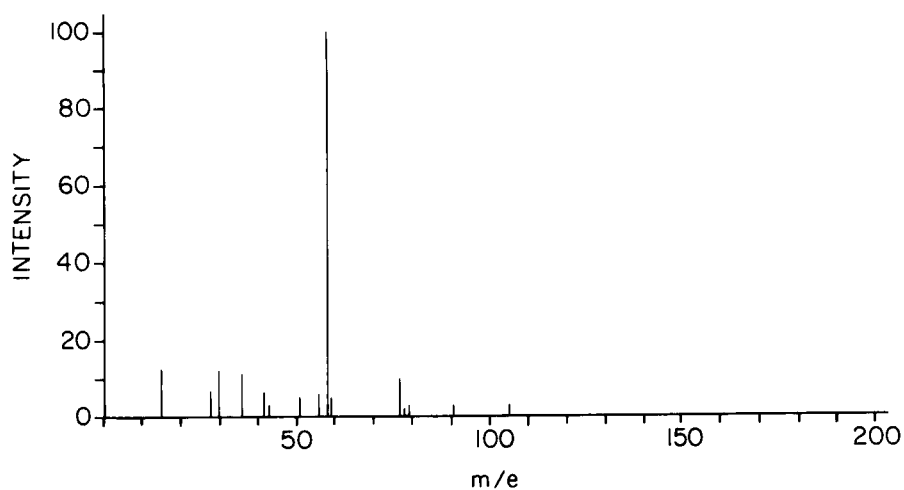


Figure 4 - Mass Spectrum of Pseudoephedrine Hydrochloride

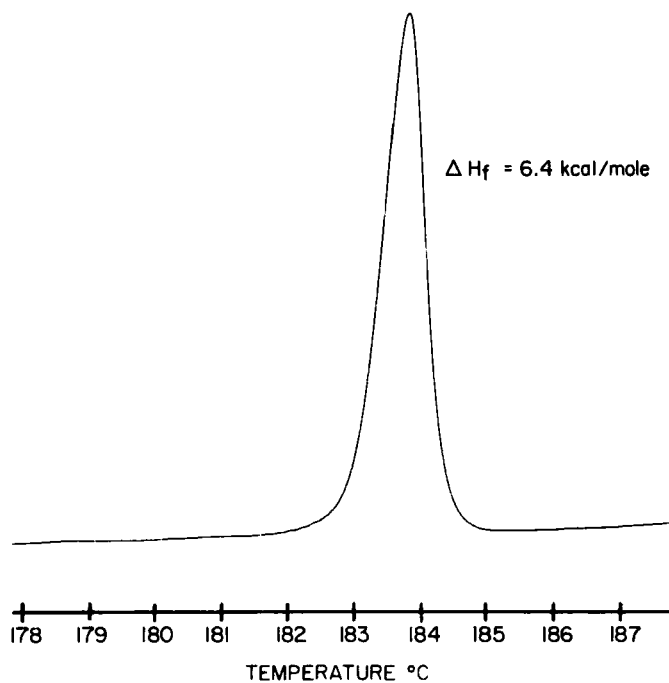


Figure 5 - DSC Curve of Pseudoephedrine Hydrochloride

various solvents at 25°C is given in Table III.¹

Table III

Solubility of Pseudoephedrine Hydrochloride at 25°C

<u>Solvent</u>	<u>Solubility (gm/ml)</u>
Water	2.0
Chloroform	0.011
Ethanol	0.278
Ether	1.4×10^{-4}

2.8 Partition Coefficient

The partition coefficients of pseudoephedrine hydrochloride at 25°C in n-octanol/aq. pH 1.2 and n-octanol/aq. pH 6.0 are 0.010 and 0.049 respectively.⁶

2.9 Differential Scanning Calorimetry (DSC)

The DSC curve of pseudoephedrine hydrochloride obtained with a Perkin Elmer DSC-1B differential scanning calorimeter is shown in Figure 5.⁷ The heating rate was 5°C/min. The heat of fusion is 6.4 Kcal/mol. The melting point (uncorrected) is 184°C.

2.10 Crystal Structure

The crystal properties of pseudoephedrine hydrochloride were determined with a GE model XRD-6 x-ray diffractometer using Zr filtered MoK_α radiation on a crystal grown from water.⁸ Pseudoephedrine hydrochloride has an orthorhombic crystal system belonging to the $P2_12_12_1$ space group. The cell dimensions are $a=25.358 \text{ \AA}$, $b=6.428 \text{ \AA}$, $c=6.901 \text{ \AA}$ with each cell containing four molecules.

2.11 Dissociation Constant

The pK_a of pseudoephedrine hydrochloride determined

titrimetrically in 80% aqueous methylcellosolve is 9.22.⁹

3. Synthesis

Pseudoephedrine hydrochloride is prepared by a Welsh rearrangement¹⁰ of *l*-ephedrine hydrochloride with acetic anhydride followed by deacetylation with hydrochloric acid.¹¹ *l*-Ephedrine can be resolved from *dl*-ephedrine with *l*-mandelic acid.¹² *l*-Ephedrine occurs naturally in certain plants of the Ma Huang species.

4. Stability

Pseudoephedrine hydrochloride can be considered a stable compound in bulk and in formulations. After 4-weeks under fluorescent lights (2400 ft. candles) and ultraviolet light (190 $\mu\text{w}/\text{cm}^2$) no discoloration or chemical degradation was observed. The bulk drug was stable for at least 6 months at 37°C and 3 months at 50°C. Tablet and syrup formulations stored at 15-30°C for 5 years showed no appreciable degradation.¹³

5. Metabolism and Pharmacokinetics

The major biotransformations of pseudoephedrine hydrochloride are parahydroxylation, N-demethylation, and oxidative deamination.¹⁴ The proposed pathways for the metabolism of pseudoephedrine are shown in Figure 6.

In a study with human subjects, whose urine pH was controlled with sodium bicarbonate and ammonium chloride, it was found that 10-25% of the administered pseudoephedrine hydrochloride was metabolized to norpseudoephedrine and the elimination of pseudoephedrine and norpseudoephedrine was related to urine pH. As the urine pH increased, the serum half-life of pseudoephedrine and norpseudoephedrine increased.¹⁵ In another similar study it was found that a decrease

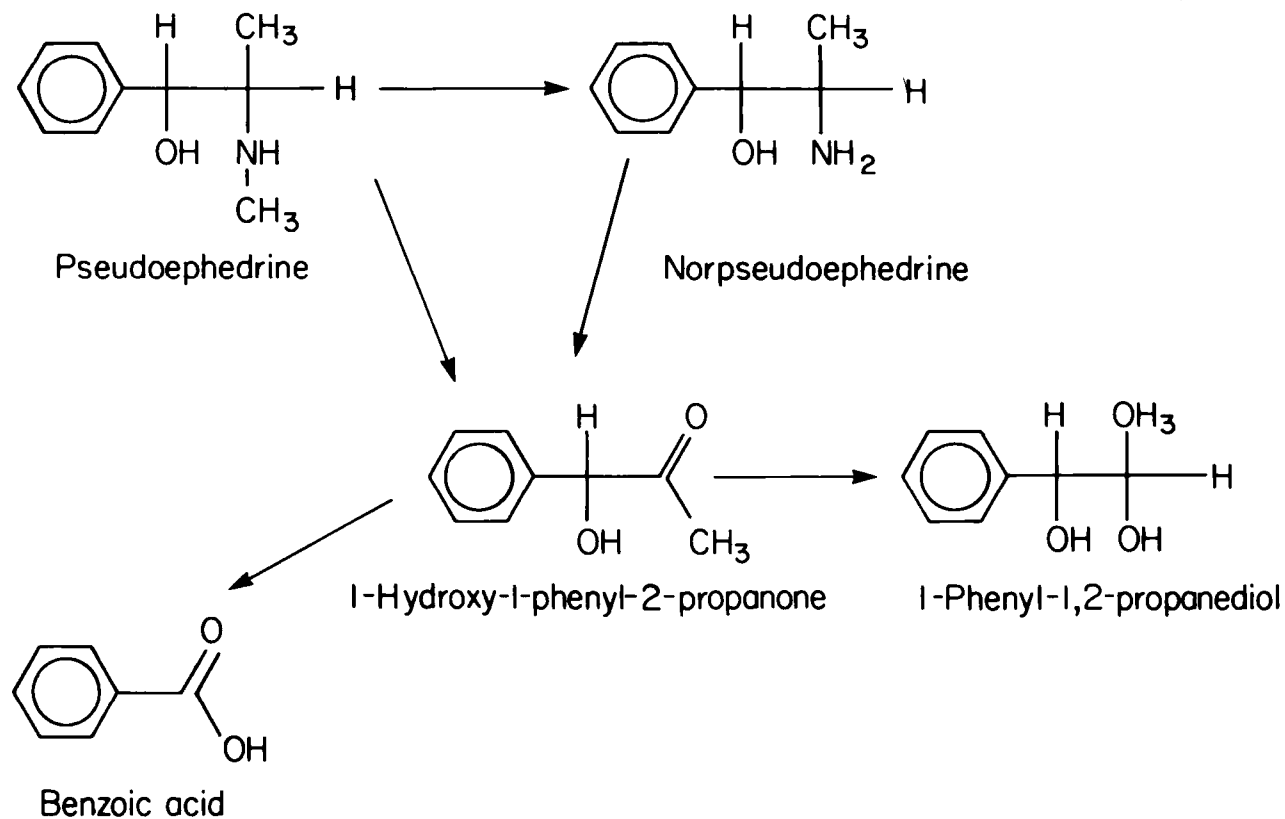


Figure 6 - Metabolism of Pseudoephedrine Hydrochloride

in urine pH caused a decrease in plasma half-life of pseudoephedrine.¹⁶ Plasma half-lives measured in normal human subjects were 5.2-8.0 hours.¹⁶

In a rat study using ¹⁴C-labelled d α -ephedrine, 85% of the i.p.-administered drug was eliminated in the first 40 hours. Two major metabolic pathways were postulated after analysis of the metabolites. The major metabolic pathway was ring para-hydroxylation forming para-hydroxyephedrine and para-hydroxynorephedrine. The minor metabolic pathway was oxidative deamination, giving acidic metabolites such as hippuric and benzoic acids.¹⁷

The relative tissue distribution in mice 15 minutes after i.v.-administered ¹⁴C-ephedrine was kidney > lung, adrenal, spleen, liver > intestines, stomach > brain, heart > plasma.¹⁷

The LD₅₀ in mice of pseudoephedrine administered i.p. is 1.0 mmole/kg.¹⁸

6. Methods of Analysis

6.1 Elemental Analysis

The results of the elemental analysis of pseudoephedrine hydrochloride are given in Table IV.⁶ The analysis was performed on a NF Reference Standard.

Table IV

Elemental Analysis of Pseudoephedrine Hydrochloride

<u>Element</u>	<u>Theory (%)</u>	<u>Found (%)</u>
C	59.55	59.54
H	8.00	8.11
N	6.95	6.81

6.2 Nonaqueous Titration

Pseudoephedrine hydrochloride is dissolved in a mixture of glacial acetic acid and mercuric acetate test solution. A standardized solution of 0.1N perchloric acid is used to titrate the solution to a blue-green end point with crystal violet indicator. Each ml of 0.1N perchloric acid is equivalent to 0.1 mmole of pseudoephedrine hydrochloride.¹

6.3 Ultraviolet Spectrophotometric Analysis

An ultraviolet spectrophotometric analysis is used to assay pseudoephedrine hydrochloride in tablets. A portion of finely powdered tablets equivalent to approximately 30 mg of pseudoephedrine hydrochloride is placed in a distilling flask which is part of a micro-steam distillation apparatus. Sodium chloride, water, and concentrated sodium hydroxide are added. A minimum of 30 ml of distillate is collected in a volumetric flask containing dilute hydrochloric acid. The flask is made to volume with distilled water and the absorbance of the solution is determined at 257 nm in 1 cm cells and compared to a solution of known concentration of NF Pseudoephedrine Hydrochloride Reference Standard.¹

An ultraviolet spectrophotometric method based on the absorbance of a periodate oxidation product of pseudoephedrine hydrochloride will be the official method of analysis in the USP XX.^{19,20} A portion of tablets or syrup in water is placed in a separatory funnel. Sodium bicarbonate and sodium metaperiodate are added. After standing for 15 minutes, 1 N HCl is added. The solution is extracted with hexane. The hexane extract is filtered and its absorbance determined at 242 nm in 1 cm cells. The amount of the oxidation product of pseudoephedrine hydrochloride is determined by comparison of the sample absorbance against the absorbance of a Pseudoephedrine Hydrochloride Reference Standard treated in the same manner.

6.4 Colorimetric Analysis

Pseudoephedrine hydrochloride in syrup formulations has been analyzed by colorimetry. Pseudoephedrine forms a stable blue-colored chelate with cupric sulfate at pH 12.5. The complex has a maximum absorbance at 500 nm. The complex is extracted from an aqueous layer with 1-pentanol. Interfering substances such as glycerine and sugars normally found in syrup formulations, which form complexes with cupric sulfate, are not extracted into 1-pentanol.²¹

6.5 Chromatography

6.51 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography has been used to analyze pseudoephedrine hydrochloride and dosage forms containing pseudoephedrine hydrochloride. Table V gives the HPLC conditions used for separations.

Table V

HPLC Conditions for Pseudoephedrine Hydrochloride

<u>Column</u>	<u>Mobile Phase</u>	<u>Retention Time (min)</u>	<u>Reference</u>
Corasil®/Phenyl	acetonitrile:	1.8 (phenyl)	22
Corasil®/C ₁₈	0.1% ammonium carbonate (9:1) pH 8.9	1.9 (C ₁₈)	
Corasil®/Phenyl	acetonitrile:	2.1 (Phenyl)	22
Corasil®/C ₁₈	1% ammonium carbonate (6:4) pH 7.4	2.2 (C ₁₈)	

Zipax®/SCX	0.02 M dibasic		
	ammonium	7	23
	phosphate:dioxane		
	(64:36)		
Nucleosil®/ silica gel	methanol:0.5M		
	sodium dihydrogen	6	24
	phosphate:		
	phosphoric acid		
	(195:50:2)		
Spherisorb®/ silica gel	ethanol:0.4%		
	ammonium	8	25
	acetate (85:15)		

6.52 Thin Layer Chromatography (TLC)

Table VI lists the various TLC systems used for pseudoephedrine hydrochloride.

Table VI

TLC Systems for Pseudoephedrine Hydrochloride

<u>Mobile Phase</u>	<u>Adsorbent</u>	<u>R_f</u>	<u>Reference</u>
ethyl acetate:	silica gel	0.25	26
cyclohexane:			
methanol:conc.			
NH ₄ OH(70:15:10:5)			
n-butanol:ethanol:	silica gel	0.46	27
water:acetic acid		0.18 (free base)	
(60:30:10:0.2)			

chloroform:	silica gel	0.33	28
methanol:			
acetone (7:3:5)		0.10 (free base)	
ethyl ether:	silica gel	0.35 (as	29
benzene (1:1)		4-chloro-	
		7-nitro-	
		benzo-2,1,3-	
		oxadiazole	
		derivative)	
chloroform:	alumina	0.70 (as	16
water:acetic		acetylated	
acid (20:75:20:1)		product)	
(lower phase)			

6.53 Paper Chromatography

Paper chromatography has been used to separate and detect pseudoephedrine hydrochloride from other pharmacologically active amines. Whatman No. 1 paper developed in n-butanol:water:95% acetic acid (4:5:1), n-butanol:toluene:water:95% acetic acid (10:10:5:5), ethyl acetate:water:95% acetic acid (3:3:1), or chloroform:water:95% acetic acid (10:5:4) gave R_f values of 0.73, 0.35, 0.57, and 0.52 for pseudoephedrine hydrochloride respectively. Visualization of pseudoephedrine hydrochloride was done by spraying the chromatogram with 0.5% bromocresol green in methanol or 0.2% ninhydrin in acetic acid:butanol 5:95.³⁰

6.54 Gas Chromatography

Pseudoephedrine hydrochloride has been separated from other amines by gas chromatography. The oxazolidine

derivative has been prepared by the reaction of pseudoephedrine with anhydrous acetone. On a 1.15% SE-30, glass column, 2.4 m x 3 mm i.d. at 104°C the oxazolidine derivative has a retention time of 16.4 minutes.³¹ On a 15% PEG 6000, glass column 2 m x 4 mm i.d. at 175°C the oxazolidine derivative had a retention time of 10.1 minutes.³²

The N-trifluoroacetyl-L-prolylchloride derivative of pseudoephedrine has retention time of 105 minutes on a 3% SE-30, stainless steel column, 2 m x 3 mm i.d. at 170°C.³³

An on-column acetic anhydride derivatization technique has been described for pseudoephedrine hydrochloride. Immediately after injection of a solution of pseudoephedrine onto a 20% SE-30, 1.8 m x 7 mm i.d. glass column at 125°C, an injection of acetic anhydride was made. The pseudoephedrine derivative formed on column has a retention time of 55.5 minutes as compared to a retention time of 8.7 minutes for underivatized pseudoephedrine.³⁴

A variety of methods have been used to determine pseudoephedrine hydrochloride levels in plasma and urine by gas chromatography. Bye and co-workers³⁵ extracted baseified plasma or urine with diethyl ether. The ether extract concentrate was chromatographed on a 1.2 m x 2mm i.d. glass column packed with 2% Carbowax 20 M +5% KOH. The column was maintained at 187°C for plasma samples and 150°C for urine samples.

The heptafluorobutyric anhydride derivative of pseudoephedrine and electron capture detector have been used to enhance the sensitivity of the gas chromatographic method. Lin and co-workers³⁶ and Cummins and Fourier³⁷ extracted baseified urine or serum with benzene. Heptafluorobutyric anhydride is added to the benzene extract. The heptafluorobutyric anhydride derivative extracted was chromatographed

on a 3% OV-17, 1.82 m x 2 mm i.d. glass column at 150°C³⁶ or a 5% ethylene glycol succinate, 1.82 m x 2 mm i.d. stainless steel column at 140°C.³⁷

Pseudoephedrine in urine was analyzed by gas chromatography using a 2% polyethylene glycol 600 + 5% KOH, 2 m x 2 mm i.d. stainless steel column at 165°C. The urine was extracted with diethyl ether and then made basic with 5N NaOH. The pseudoephedrine was extracted with diethyl ether, concentrated, and injected directly into the gas chromatograph, or derivatized with acetone and then chromatographed.³⁸

Pseudoephedrine was determined after acidification, precipitation, and acetic anhydride derivatization. The ester derivative was injected onto a 2.5% SE-30, 1.8 m x 4 mm i.d. column at 190°C.³⁹

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TRIPROLIDINE HYDROCHLORIDE

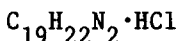
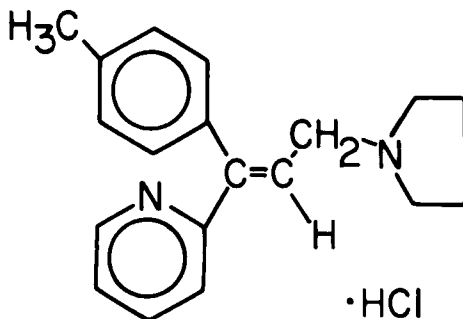
Steven A. Benezra and Chen-Hwa Yang

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 - 6.52 Thin-Layer Chromatography
 - 6.53 Quantitative Thin-Layer Chromatography
 - 6.54 Gas Chromatography
 - 6.6 Fluorimetric Analysis

1. Description

1.1 Name, Formula, Molecular Weight

Triprolidine hydrochloride is E-2-[3-(1-pyrrolidinyl)-1-p-tolylpropenyl]pyridine, monohydrochloride; E-1-(2-pyridyl)-3-pyrrolidino-1-p-tolylprop-1-ene, monohydrochloride.



M.W. 314.85

1.2 Appearance, Color, Odor

Triprolidine hydrochloride occurs as a white, crystalline powder, with no more than a slight unpleasant odor.¹

2. Physical Properties

2.1 Infrared Spectrum (IR)

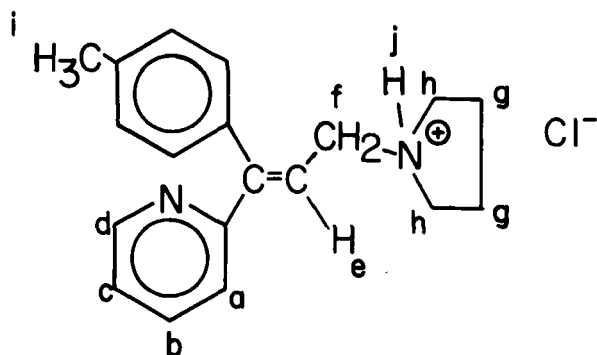
The infrared spectrum of triprolidine hydrochloride is shown in Figure 1. The IR spectrum was recorded with a Nicolet model 7199 FT-IR spectrophotometer as a 0.2% dispersion of triprolidine hydrochloride in KBr.² Table I gives the infrared assignments consistent with the structure of triprolidine hydrochloride.

Table I
Infrared Spectral Assignments for Triprolidine
Hydrochloride Monohydrate

<u>Band (cm⁻¹)</u>	<u>Assignment</u>
3480	OH stretch (hydrate)
2958	CH stretch (-CH ₃)
2690	NH ⁺ stretch
1630	C=C stretch (conj. diene)
1582	C=C stretch (aromatic)
1562	C=C stretch (pyridine)
1462	-CH ₃ asym. bend
1386	-CH ₃ sym. bend
1358	C-N stretch (tert. amine)
846	=C-H rock
824	para subst. benzene
776	1-subst. pyridine

2.2 Nuclear Magnetic Resonance (NMR) Spectrum

The NMR spectrum of triprolidine hydrochloride is shown in Figure 2. It was obtained with a Varian XL-100 100 MHz NMR spectrometer. Deuterated DMSO was used as the solvent with tetramethylsilane as an internal standard.³ The interpretation of the NMR spectrum is given in Table II.



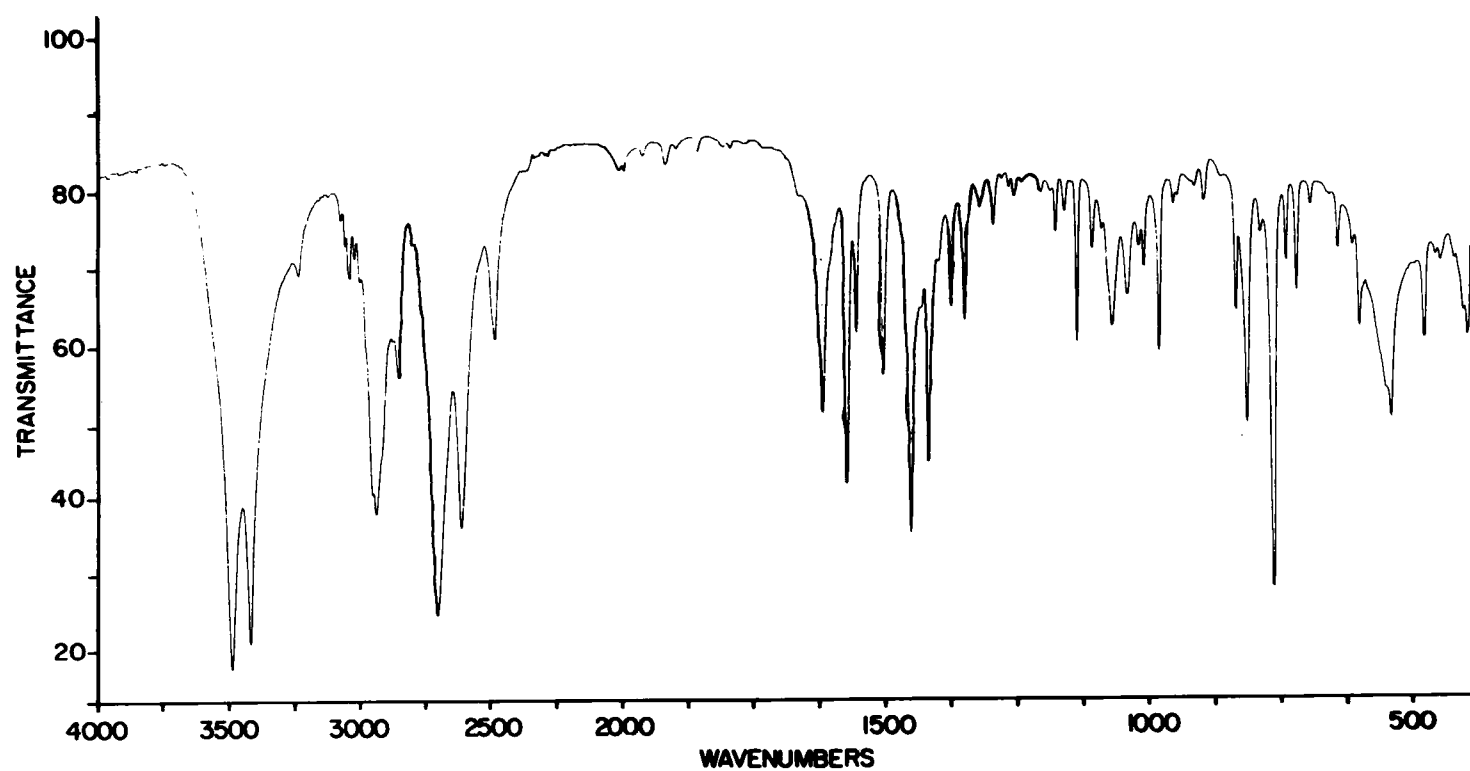


Figure 1 - Infrared Spectrum of Triprolidine Hydrochloride

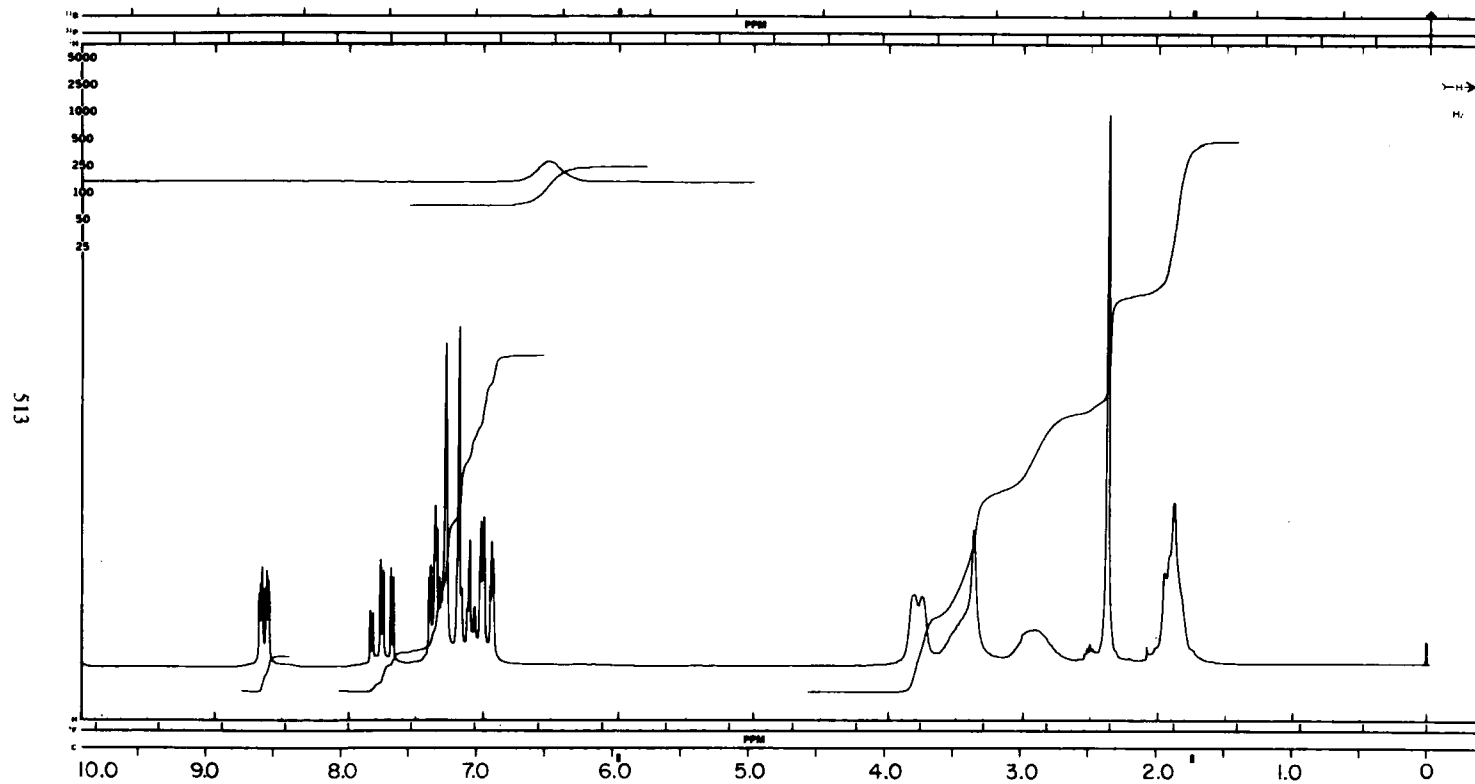


Figure 2 - Nuclear Magnetic Resonance Spectrum Triprolidine Hydrochloride

Table IINMR Assignments for Triprolidine Hydrochloride

<u>Chemical Shift (ppm)</u>	<u>No. of Protons</u>	<u>Multiplicity</u>	<u>Assignments</u>
11.55	1	1	j
8.63	1	multiplet	a
7.74	1	6	b
7.35	1	multiplet	c
7.00	1	multiplet	d
7.12-7.38	4	4	benzene ring
6.95	1	3	e
3.78	2	2	f
3.49	2	1 (broad)	h
2.94	2	1 (broad)	h
2.37	3	1	i
1.88	4	multiplet	g

$$J_{a,c}=4.7, J_{a,d}=0.9, J_{a,b}=2.0, J_{b,c}=8, J_{c,d}=1.2, J_{af}=7$$

2.3 Ultraviolet (UV) Spectrum

The ultraviolet spectrum of triprolidine hydrochloride in 0.1N HCl was taken with a Beckman ACTA CIII UV spectrophotometer and is shown in Figure 3.⁴ Table III gives the UV data for triprolidine hydrochloride in various solvents.

Table IIIUV Spectral Data for Triprolidine Hydrochloride

<u>Solvent</u>	<u>λ_{\max} (nm)</u>	<u>ϵ_{\max}</u>
0.1N NaOH	234	17000
	275	8000
0.1N HCl	232	13000
	290	9900
Methanol	235	15000
	282	7200

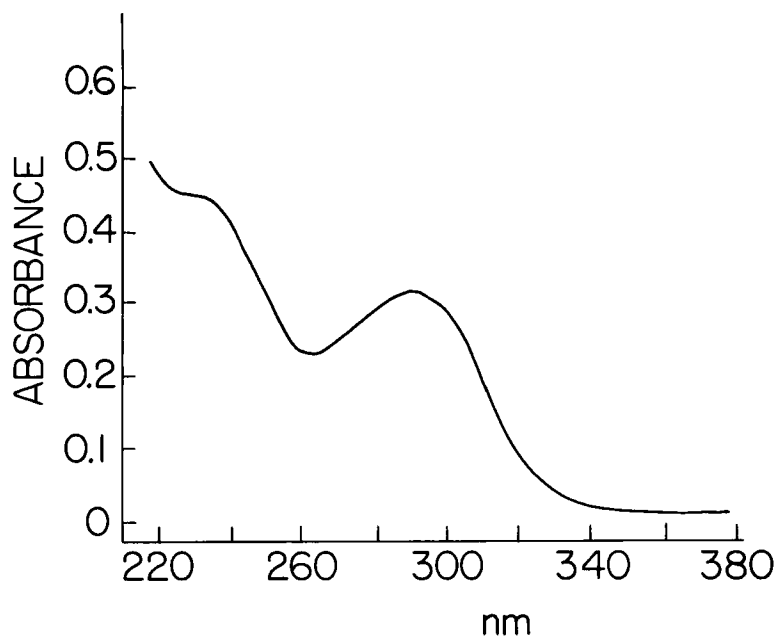


Figure 3 - Ultraviolet Spectrum of Triprolidine Hydrochloride

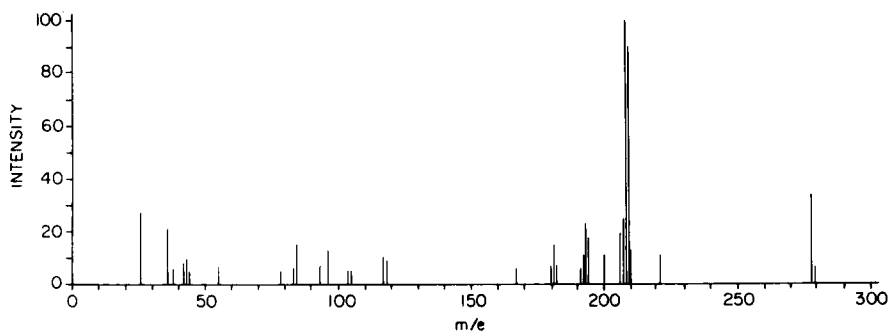


Figure 4 - Mass Spectrum of Triprolidine Hydrochloride

2.4 Mass Spectrum

The mass spectrum of triprolidine hydrochloride is shown in Figure 4. It was obtained with a Varian MAT CH5-DF mass spectrometer. The electron energy was 70 eV and the sample was introduced via direct probe at 120°C.⁵ The characteristic fragments are in agreement with those found by Kuntzman and co-workers.⁶ The major fragments characteristic of triprolidine are shown in Figure 5.

2.5 Fluorescence Spectrum

Triprolidine hydrochloride in 0.1M sulfuric acid has an excitation maximum at 305 nm and an emission maximum at 445 nm. The fluorescence is much diminished in hydrochloric acid and almost non-existent in distilled water. The addition of halide ions to 0.1M sulfuric acid solutions of triprolidine hydrochloride has a quenching effect on the fluorescent intensity. The degree of quenching is $I^- > Br^- > Cl^- > F^-$. A concentration of $10^{-3} M Cl^-$ has little effect on the fluorescent intensity, while 0.1M Cl^- reduces the intensity by approximately 75% of the chloride-free sulfuric acid solution.

2.6 Crystal and Molecular Structure

James and Williams have determined that triprolidine hydrochloride monohydrate crystals belong to the $P2_1/c$ space group.⁸ There are 4 molecules per unit cell. The cell parameters are $a = 14.777 \text{ \AA}$, $b = 9.5785 \text{ \AA}$, $c = 13.099 \text{ \AA}$, and $\beta = 90.48^\circ$. The 2-pyridyl ring and p-tolyl group make dihedral angles of 29.7° and 55.3° respectively with the double bond plane. The inter-aryl dihedral angle is 106.5° . The data was obtained on crystals grown from a solution of the compound in anisole. A Picker FACS-1 diffractometer with CuK_α radiation was used for the measurements.

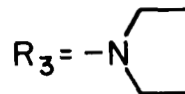
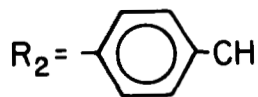
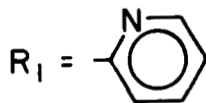
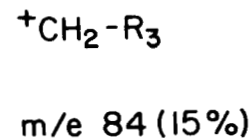
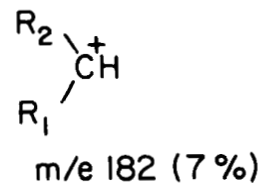
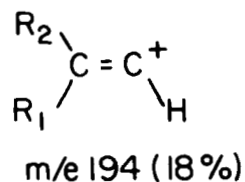
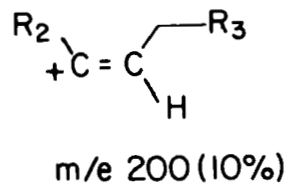
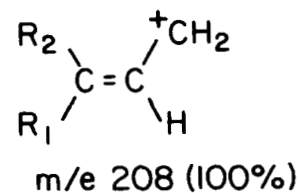
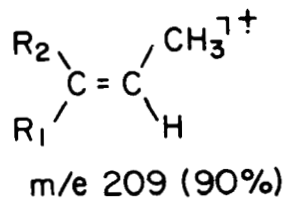
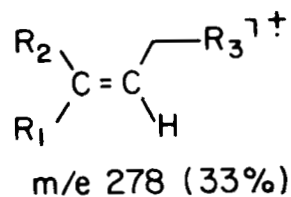


Figure 5 - Major Ions in Mass Spectrum of Triprolidine Hydrochloride

2.7 Melting Point

Triprolidine hydrochloride melts at 115-120°C in a sealed capillary tube.⁹

2.8 Solubility

The solubility of triprolidine hydrochloride, as the monohydrate, in various solvents at 25°C is given in Table IV.⁹

Table IV
Solubility of Triprolidine Hydrochloride at 25°C

<u>Solvent</u>	<u>Solubility (mg/ml)</u>
Water	316
0.1N HCl	319
95% Ethanol	374
n-Octanol	75
Chloroform	480
Propylene Glycol	237
Diethyl Ether	<1

2.9 Partition Coefficients

The partition coefficients of triprolidine hydrochloride in n-octanol/aqueous pH 1.2 and n-octanol/aqueous pH 7.4 are 0.041 and 7.0 respectively.⁹

2.10 Dissociation Constant

The pK_{a1} and pK_{a2} of triprolidine are 3.6 and 9.3 respectively.⁹

3. Synthesis

The synthesis of triprolidine hydrochloride is shown in Figure 6.¹⁰ The 4-methyl-*w*-pyrrolidinopropiophenone used in the first step is prepared by the Mannich

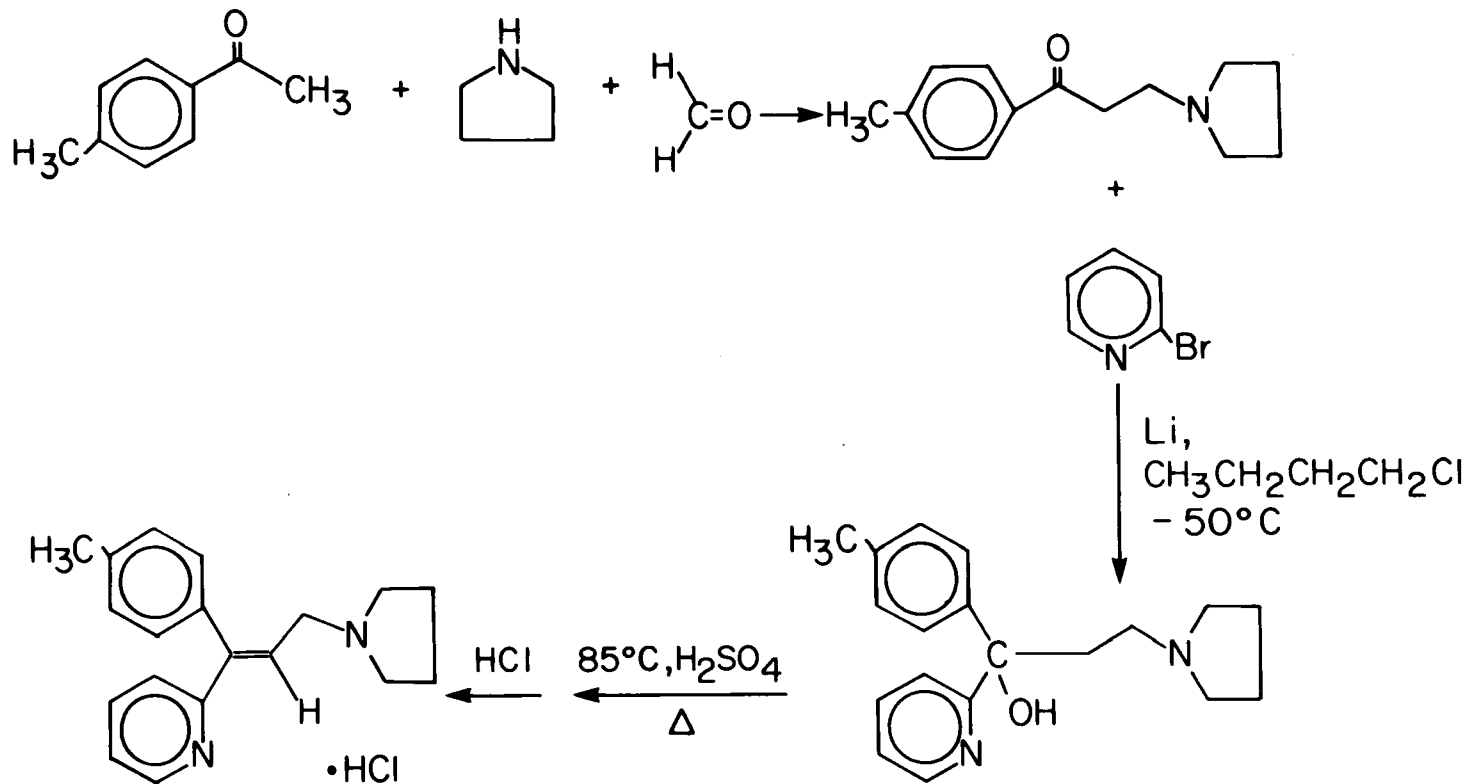


Figure 6 - Synthesis of Triprolidine Hydrochloride

reaction of 4-methylacetophenone and pyrrolidine. With proper dehydration conditions of the carbinol, a product almost pure in the desired E-conformation is obtained.

4. Stability

Ultraviolet light will convert the E-form of triprolidine hydrochloride to the Z-form. After 2 years at 37°C, triprolidine hydrochloride in a syrup formulation does not decompose more than 10%. After 3 years at 37°C triprolidine hydrochloride in tablet formulations does not decompose more than 10%. The formulations are kept in light resistant containers since triprolidine hydrochloride discolors on exposure to light.⁹

5. Drug Metabolic Products and Pharmacokinetics

Figure 7 shows the major metabolic pathway of triprolidine hydrochloride as determined in a study of ¹⁴C labelled triprolidine hydrochloride in guinea pigs.⁶ Triprolidine is converted to metabolite I by liver microsomes. Metabolite I is converted to metabolite II which is the major metabolite found in guinea pig urine.

The plasma levels of triprolidine hydrochloride were determined in 16 normal male subjects.¹² When administered orally at a concentration of 3.75 mg triprolidine hydrochloride in 15 ml of syrup, peak plasma levels of 8.2 ng/ml were achieved in 2 hours with a drug half-life of 5 hours. The low plasma levels found indicate a large volume of tissue distribution which was consistent with data obtained from rat studies.

6. Methods of Analysis

6.1 Elemental Analysis

The results of the elemental analysis of triprolidine hydrochloride are given in Table V.³ The theoretical

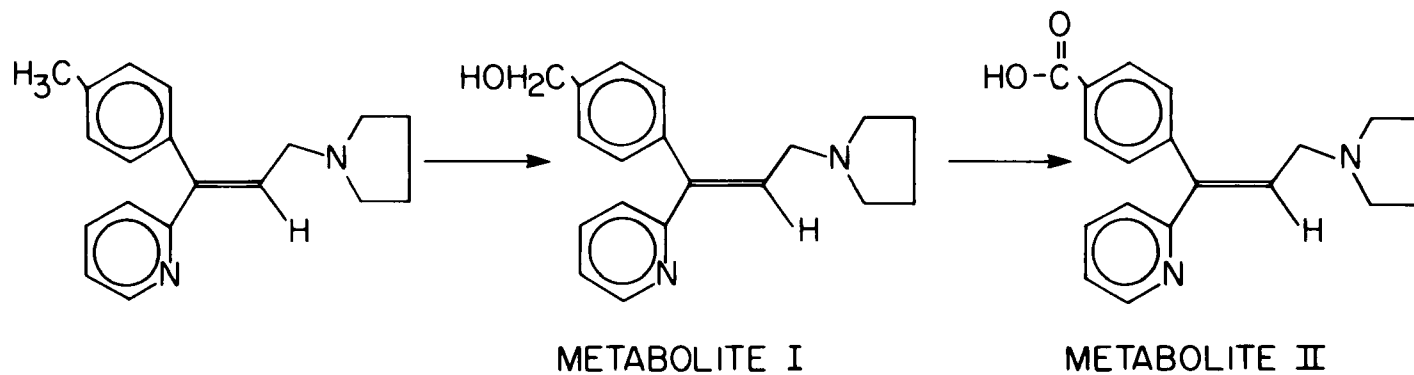


Figure 7 - Metabolites of Triprolidine Hydrochloride

figures are based on the monohydrate.

Table V

Elemental Analysis of Triprolidine Hydrochloride

<u>Element</u>	<u>Theory (%)</u>	<u>Found (%)</u> *
C	58.50	58.59
H	7.51	7.58
N	8.41	8.39

*N.F. Reference Standard

6.2 Nonaqueous Titration

Triprolidine hydrochloride is dissolved, with warming if necessary, in glacial acetic acid. Mercuric acetate test solution is added and the solution titrated with 0.1N perchloric acid, the end-point being determined potentiometrically. Each ml. of 0.1N perchloric acid is equivalent to 0.05 mmole of triprolidine hydrochloride.¹

6.3 Ultraviolet Spectrophotometric Analysis

An ultraviolet spectrophotometric analysis is used to determine content uniformity of triprolidine hydrochloride in tablet formulations.¹ The triprolidine hydrochloride is extracted from finely powdered tablets with dilute hydrochloric acid. The solution is filtered and diluted to a concentration of approximately 10 µg triprolidine hydrochloride per ml. The absorbance at 290 nm of the extracted triprolidine hydrochloride solution in 1 cm cells is compared against NF Triprolidine Hydrochloride Reference Standard prepared in dilute hydrochloric acid at the 10 µg/ml level.

6.4 Quantitative Infrared Analysis

Quantitative infrared analysis is used as an assay

procedure for triprolidine hydrochloride in syrup and tablets.¹ A portion of syrup or tablets equivalent to 20 mg and 12.5 mg triprolidine hydrochloride, respectively, is placed in a separatory funnel or glass-stoppered test tube. Distilled water is added to dilute the sample followed by concentrated NaOH solution to liberate triprolidine free base. The triprolidine free base is extracted into cyclohexane. The infrared absorbance of the cyclohexane extract is determined at 824 cm^{-1} in 1 mm thick cells. A base line is drawn between 840 cm^{-1} and 806 cm^{-1} . The absorbance of the sample is compared against the absorbance of a NF Triprolidine Hydrochloride Reference Standard treated in the same manner as the sample.

6.5 Chromatography

6.51 High Performance Liquid Chromatography (HPLC)

Separation of the E and Z isomers of triprolidine hydrochloride has been performed with HPLC. A DuPont SAX column (1m x 2 mm i.d.) with a mobile phase of 0.04M NaNO_3 and 0.01M Na_2HPO_4 at a pressure of 1500 psi gave retention times of 4 minutes and 9 minutes for the E and Z isomers respectively.¹³ A 0.45m x 4 mm i.d. Partisil® silica gel column with a mobile phase of acetonitrile:concentrated $\text{NH}_4\text{OH}:\text{H}_2\text{O}$ (1000:5:45) at 2 ml/min gave retention times of 15 and 22 minutes for E and Z isomers respectively.¹⁴ A mixture of triprolidine hydrochloride with other drugs normally found in combination with triprolidine hydrochloride was separated with a 1.22m x 2.3 mm i.d. column containing a chemically bonded diphenyldichlorosilane pellicular packing. A mobile phase consisting of 60:40 acetonitrile:1% aqueous ammonium acetate (pH 7.4) with a flow rate of 1.4 ml/min gave a retention time of 310 seconds for triprolidine.¹⁵ Syrup and

tablet formulations of triprolidine hydrochloride can be analyzed by HPLC using a Spherisorb® 10µ C₁₈ reversed phase column eluted with ethanol:0.5% aqueous ammonium acetate (4:1) at 2 ml/min. In this system triprolidine hydrochloride has a retention time of 22 minutes.¹⁶

6.52 Thin Layer Chromatography (TLC)

Various TLC systems for triprolidine hydrochloride are given in Table VI.

Table VI

Thin Layer Chromatography Systems for Triprolidine Hydrochloride

<u>Mobile Phase</u>	<u>Adsorbant</u>	<u>Rf</u>	<u>Reference</u>
chloroform:methanol:ammonia 80:20:1	Silica gel F	0.60	17
chloroform:diethylamine 95:5	Silica gel F	0.44	17
2-butanone:dimethylformamide 1:1	Silica gel F	0.60	17
n-butylacetate:acetone: n-butanol:methanol:10% aq. ammonia 4:2:2:1:1	Silica gel F	0.60	17
2-butanone:toluene: methanol:diethylamine 60:40:7:3	Silica gel F	0.40	17
cyclohexane:benzene: diethylamine 75:15:10	Silica gel G	0.41	18
methanol	Silica gel G pre-coated with	0.40	18

	0.1M NaOH		
acetone	Silica gel G	0.13	19
	pre-coated with		
	0.1M NaOH		
methanol	Silica gel G	0.17	18
	pre-coated		
	with 0.1M KHSO ₄		
95% ethanol	Silica gel G	0.02	19
	pre-coated with		
	0.1M KHSO ₄		
benzene:dioxane:ammonia	Silica gel G	0.40	20
60:35:35			
ethanol:acetic acid:water	Silica gel G	0.48	20
5:3:2			
methanol:n-butanol	Silica gel G	0.26	20
6:4			
n-butanol:n-butyl ether:	Alumina	0.81	20
acetic acid			
4:8:1			

6.53 Quantitative Thin Layer Chromatography

Quantitative TLC has been used to determine triprolidine hydrochloride in human plasma.¹² Plasma was extracted with dichloroethane at physiological pH (7.4). The organic phase was evaporated and the residue dissolved in chloroform. A portion of the chloroform solution was spotted on silica gel plates which were then developed in chloroform:methanol:ammonia (89:10:1). After development, the plate was air dried and sprayed with a 2M aqueous solution of ammonium bisulfate. After the plate was air dried for 1 hour, the fluorescent spot representing triprolidine was quantitated with a spectrodensitometer in the reflectance mode with 300 nm excitation and emission above 405 nm. With

this technique it was possible to quantitate as little as 0.4 ng triprolidine spotted on the plate.

6.54 Gas Chromatography

Triprolidine hydrochloride has been separated from other antihistamines with a 1.52m x 2.4 mm i.d. column packed with 2% carbowax 20M + 10% KOH on 60/80 mesh Chromosorb W. Retention time of 32 minutes was obtained with a column temperature of 190°C. Triprolidine hydrochloride was probably eluted as the free base.²¹

6.6 Fluorimetric Analysis

Triprolidine hydrochloride in syrups and tablets can be analyzed by fluorimetry. A portion of the tablets or syrup is made basic with 1N NaOH and extracted with ethylene chloride. The organic phase is then extracted with 0.1N H₂SO₄. The fluorescence of the acid extract is determined with a fluorometer using a UG11 filter for excitation and a Wratten 2A filter for emission. The fluorescence of the sample preparation is compared against a Reference Standard prepared in the same manner²².

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SODIUM VALPROATE AND VALPROIC ACID

Zui L. Chang

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 - 1.12 Trade Names
 - 1.2 Formulas and Molecular Weights
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1. Description

1.1 Nomenclature

1.11 Chemical Names

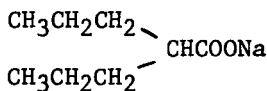
Sodium valproate is sodium 2-propylpentanoate. It is also known as sodium dipropylacetic acid, sodium 2-propylvalerate, sodium dipropylacetate, sodium di-n-propylacetate, and by many slight variations of the particular nomenclature.

Valproic acid is 2-propylpentanoic acid. It is also known as n-dipropylacetic acid, 2-propylvaleric acid, DPA, and by many slight variations of the particular nomenclature.

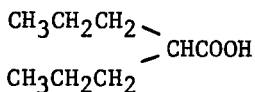
1.12 Trade Names

Depakene®, Depakine®, Eureka®, Epilim®, Ergenyl®, and Labazene®.

1.2 Formulas and Molecular Weights



$\text{C}_8\text{H}_{15}\text{O}_2\text{Na}$ M.W. 166.19



$\text{C}_8\text{H}_{16}\text{O}_2$ M.W. 144.21

1.3 Appearance, Color, Odor

Sodium valproate is a white crystalline powder with a very slight characteristic odor of valproic acid.

Valproic acid is a colorless slightly viscous liquid, and it has a characteristic odor of valproic acid.

2. Physical Properties

2.1 Infrared Spectra

The infrared spectrum of sodium valproate is presented in Figure 1. The spectrum was measured in the solid state as a potassium bromide dispersion. The following bands (cm^{-1}) have been assigned for Figure 1 (1).

- a. 3000-2800 cm^{-1} Complex of strong bands due to the overlapping C-H stretching vibrations of the various methyl and methylene groups.
- b. 1560 and 1412 cm^{-1} Strong bands due to the anti-symmetrical and symmetrical stretching vibrations of the COO^- grouping.

The infrared spectrum of valproic acid is presented in Figure 2. The spectrum was measured using the capillary method. The following bands (cm^{-1}) have been assigned for Figure 2 (1).

- a. 3400-2300 cm^{-1} Broad and diffuse absorption due to the OH stretching vibration of the carboxylic acid.
- b. 3000-2800 cm^{-1} Complex of strong bands due to the overlapping C-H stretching vibrations of the various methyl and methylene groups.
- c. 1700 cm^{-1} Strong band due to the C=O stretching vibration of the carboxylic acid.
- d. 930 cm^{-1} Broad band due to the OH bending vibration of the carboxylic acid.

2.2 Nuclear Magnetic Resonance Spectra (NMR)

The nuclear magnetic resonance spectrum of sodium valproate as shown in Figure 3 was obtained on a Varian Associates T-60 NMR Spectrometer in deuterium oxide containing tetramethylsilane as the internal standard. The spectral peak assignments (2) are presented in Table I.

FIGURE 1 - INFRARED SPECTRUM OF SODIUM VALPROATE

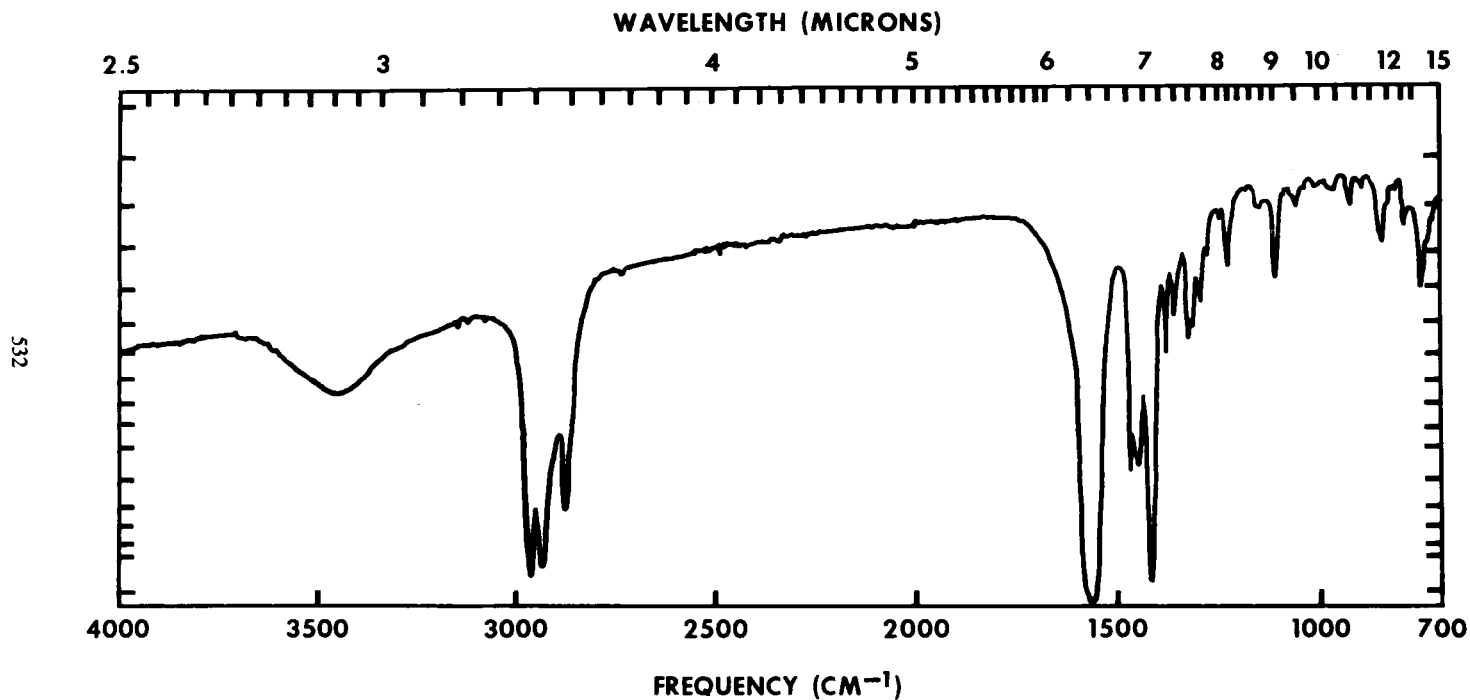
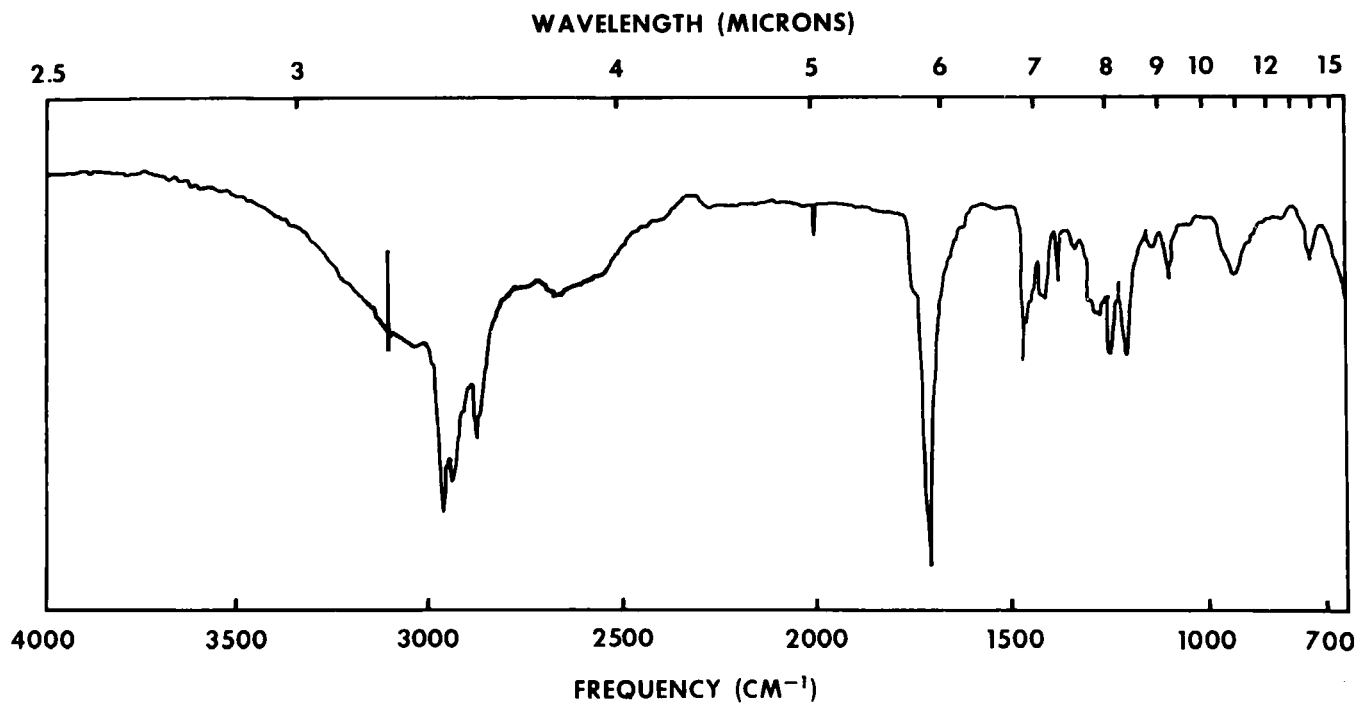


FIGURE 2 - INFRARED SPECTRUM OF VALPROIC ACID



**FIGURE 3 - NUCLEAR MAGNETIC RESONANCE SPECTRUM
OF SODIUM VALPROATE**

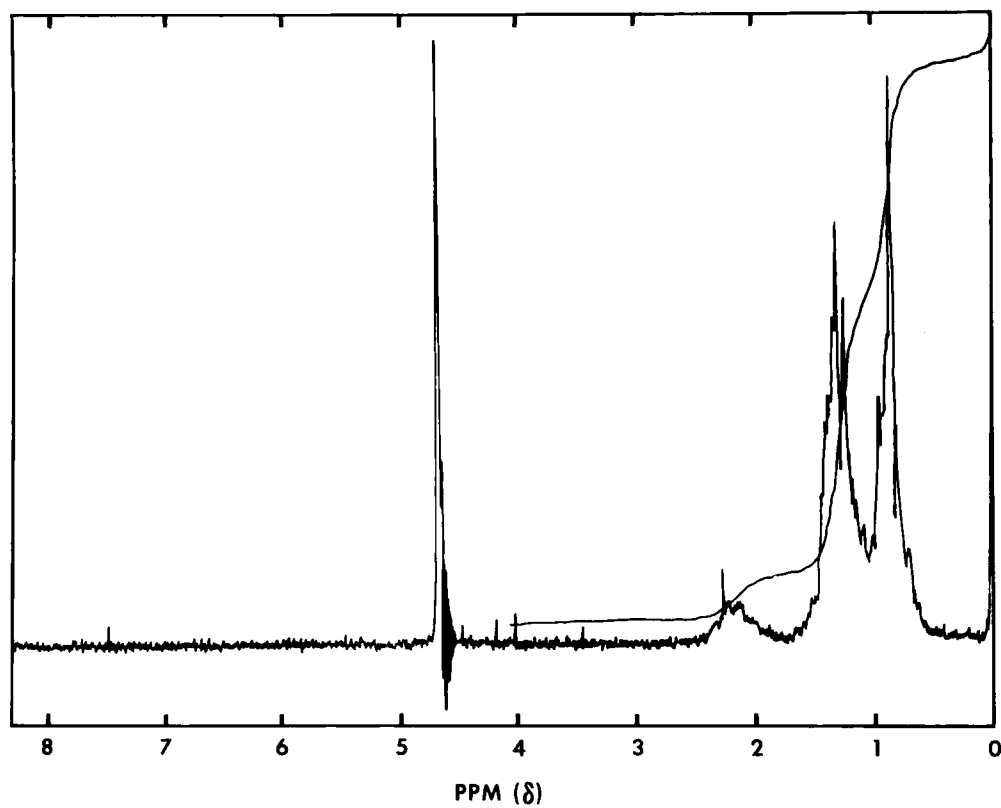


Table I

NMR Spectral Assignments for Sodium Valproate

<u>Proton Assignment</u>	<u>Chemical Shift (ppm)</u>	<u>Multiplicity</u>
>CH-CO	1.9-2.5	Multiplet
$\begin{array}{c} \text{-CH}_2\text{CH}_2 \\ \quad \diagup \\ \quad \text{C} \\ \quad \diagdown \\ \text{-CH}_2\text{CH}_2 \end{array}$	1.1-1.8	Complex
$\text{CH}_3\text{-}$	0.5-1.1	Complex

The nuclear magnetic resonance spectrum of valproic acid as shown in Figure 4 was obtained on a Varian Associates T-60 NMR Spectrometer as a 10% w/v solution in a solvent of deuterated chloroform. The spectral peak assignments (2) are presented in Table II.

Table II

NMR Spectral Assignments for Valproic Acid

<u>Proton Assignment</u>	<u>Chemical Shift (ppm)</u>	<u>Multiplicity</u>
CO_2H	11.3-11.6	Singlet
CHCO	1.9-2.5	Multiplet
$\begin{array}{c} \text{-CH}_2\text{CH}_2 \\ \quad \diagup \\ \quad \text{C} \\ \quad \diagdown \\ \text{-CH}_2\text{CH}_2 \end{array}$	1.1-1.8	Complex
$\text{CH}_3\text{-}$	0.5-1.1	Complex

2.3 Mass Spectrum

Sodium valproate was not sufficiently volatile for mass spectral analysis. The mass spectrum of valproic acid as shown in Figure 5 was obtained using an Associated Electrical Industries Model MS-902 Mass Spectrometer with the ionization electron beam energy at 70 eV. High resolution data were compiled and tabulated with the aid of an on-line PDP-11 Computer.

Valproic acid was quite volatile and vaporized as soon as it was admitted to the source of the mass spectrometer. Only a very weak ion was detectable in the molecular ion region at m/e 145. This would correspond to $(M+H)^+$, but exact mass measurement was not possible because of the peak's small size and the short lifetime of the sample in the mass spectrometer.

The mass spectrum assignments of the prominent ions and subsequent fragments are shown in Table III and Figure 6 (3).

Table III

High Resolution Mass Spectrum of Valproic Acid

<u>Measured Mass (m/e)</u>	<u>Calculated Mass</u>	<u>Formula</u>
126.1044	126.1045	$C_8H_{14}O$
102.0690	102.0681	$C_5H_{10}O_2$
73.0295	73.0290	$C_3H_5O_2$

2.4 Raman Spectra

The Raman spectrum of sodium valproate as shown in Figure 7 was obtained in the solid state on a Cary Model 83 Spectrometer. The following bands (cm^{-1}) have been assigned for Figure 7 (1).

- a. 3000-2800 cm^{-1} Complex of strong bands due to the overlapping C-H stretching vibrations of the various methyl and methylene groups.
- b. 1450 cm^{-1} Due to the superimposing C-H bending vibrations of the various methyl and methylene groups.

The Raman spectrum of valproic acid as shown in Figure 8, was obtained in the undiluted liquid state on a Cary Model 83 Spectrometer. The following bands (cm^{-1}) have been assigned for Figure 8 (1).

**FIGURE 4 - NUCLEAR MAGNETIC RESONANCE SPECTRUM
OF VALPROIC ACID**

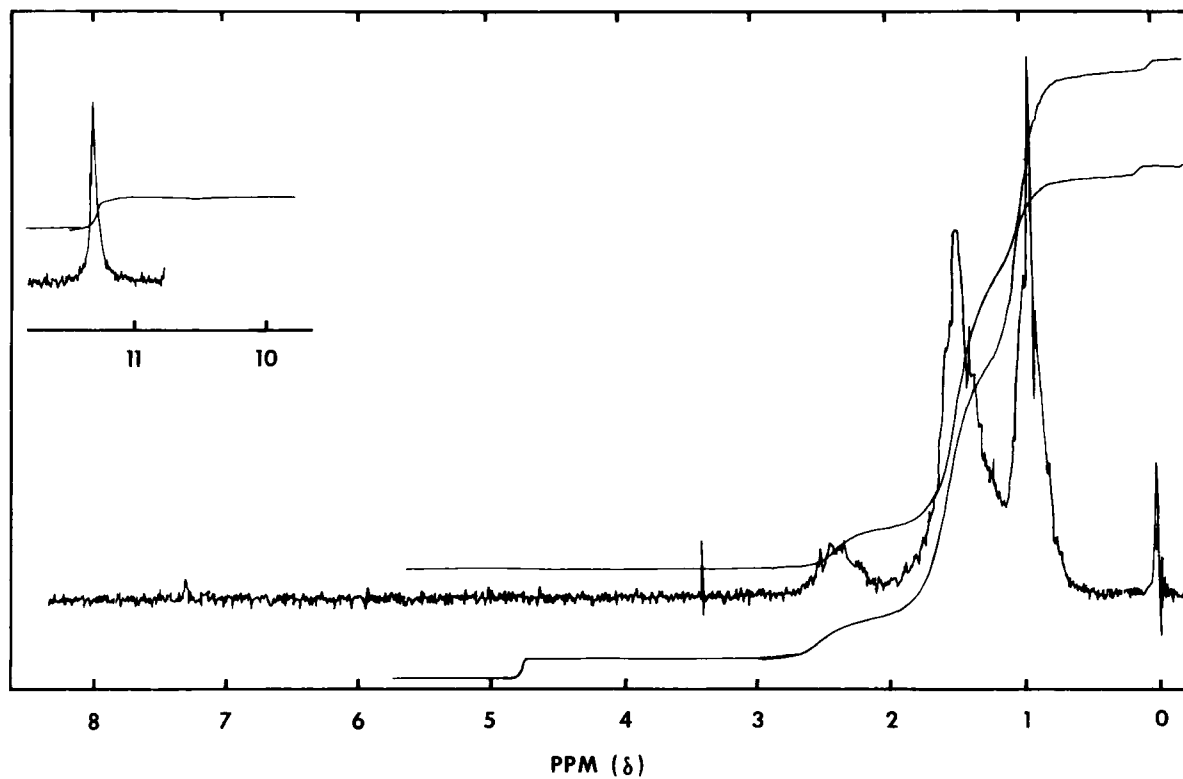


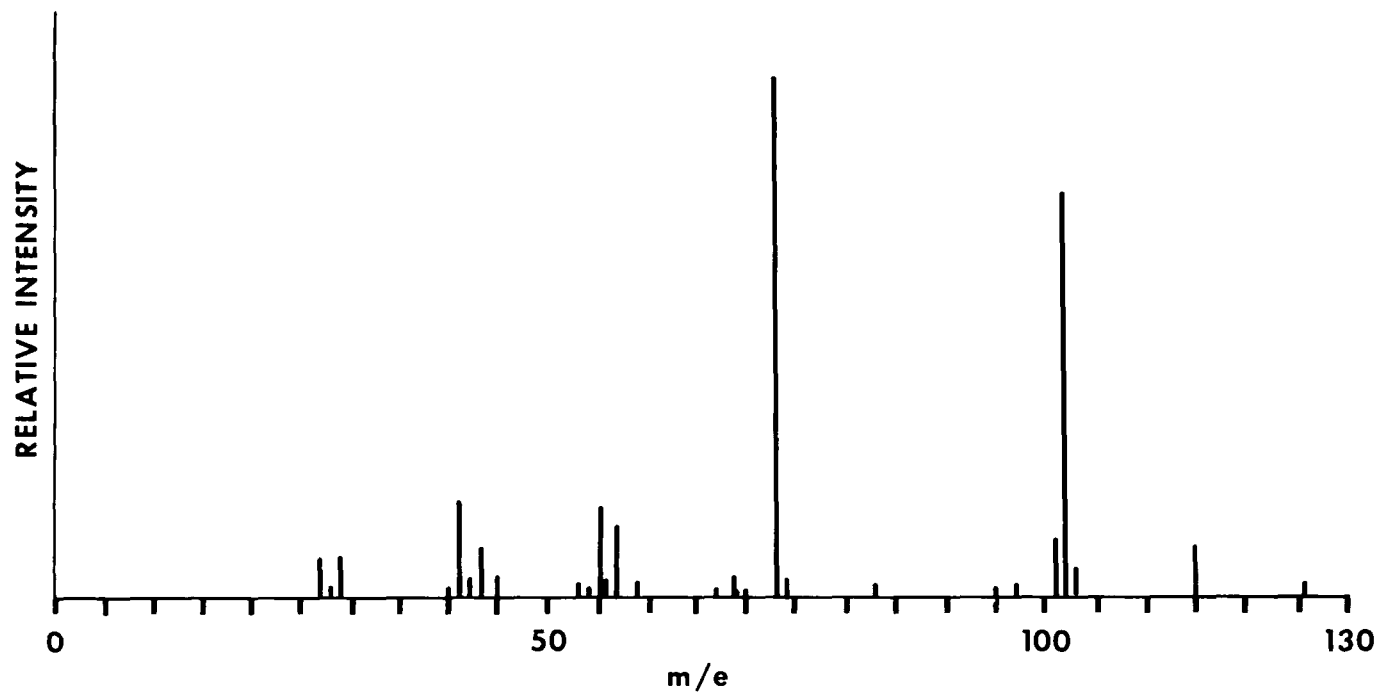
FIGURE 5 - MASS SPECTRUM OF VALPROIC ACID

FIGURE 6 - FRAGMENTATION PATHWAYS OF VALPROIC ACID

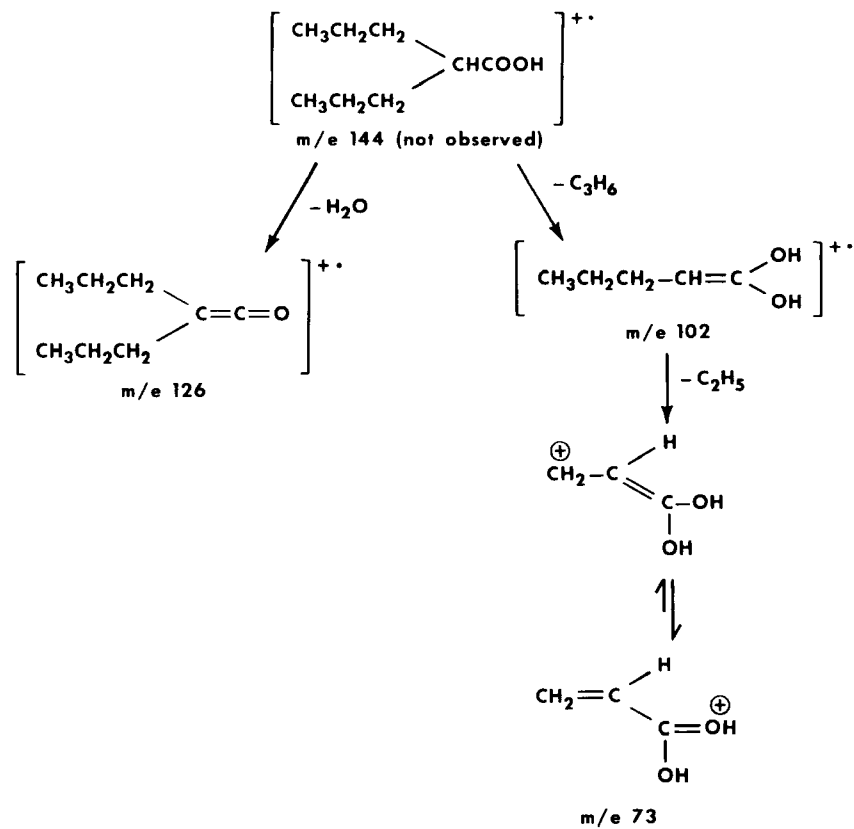


FIGURE 7 - RAMAN SPECTRUM OF SODIUM VALPROATE

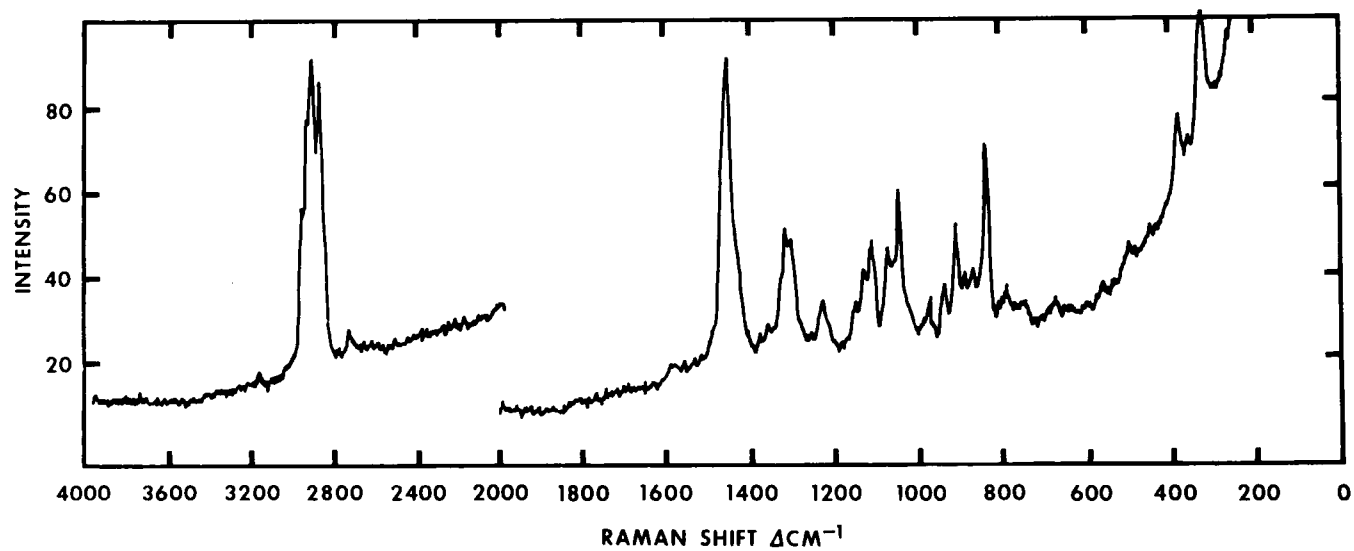
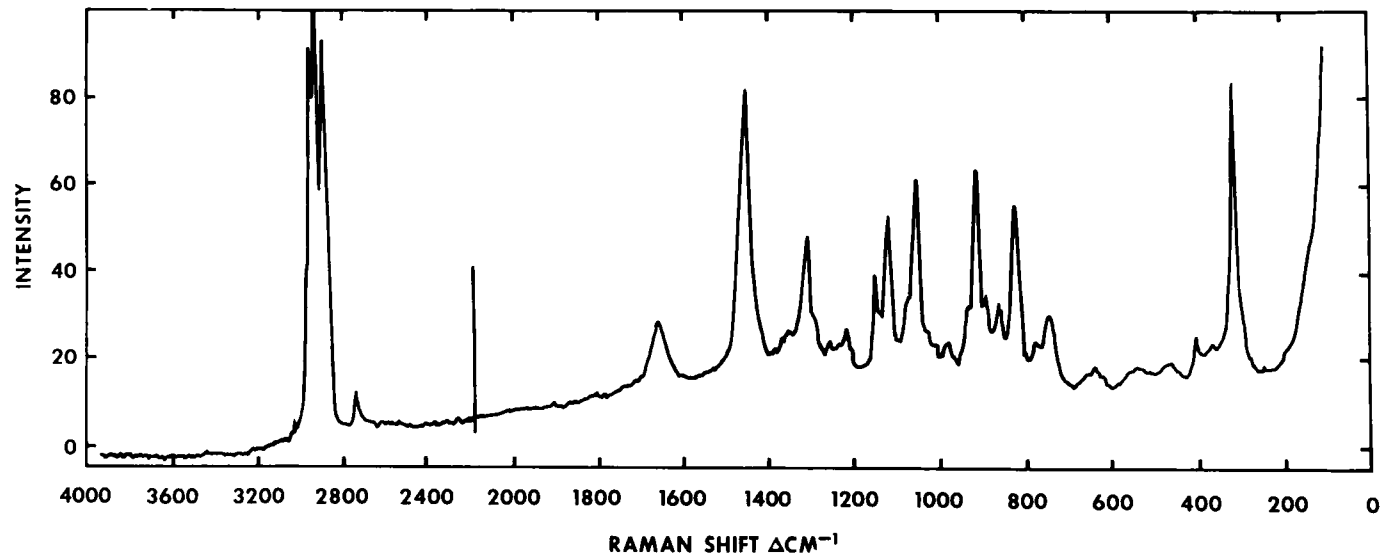
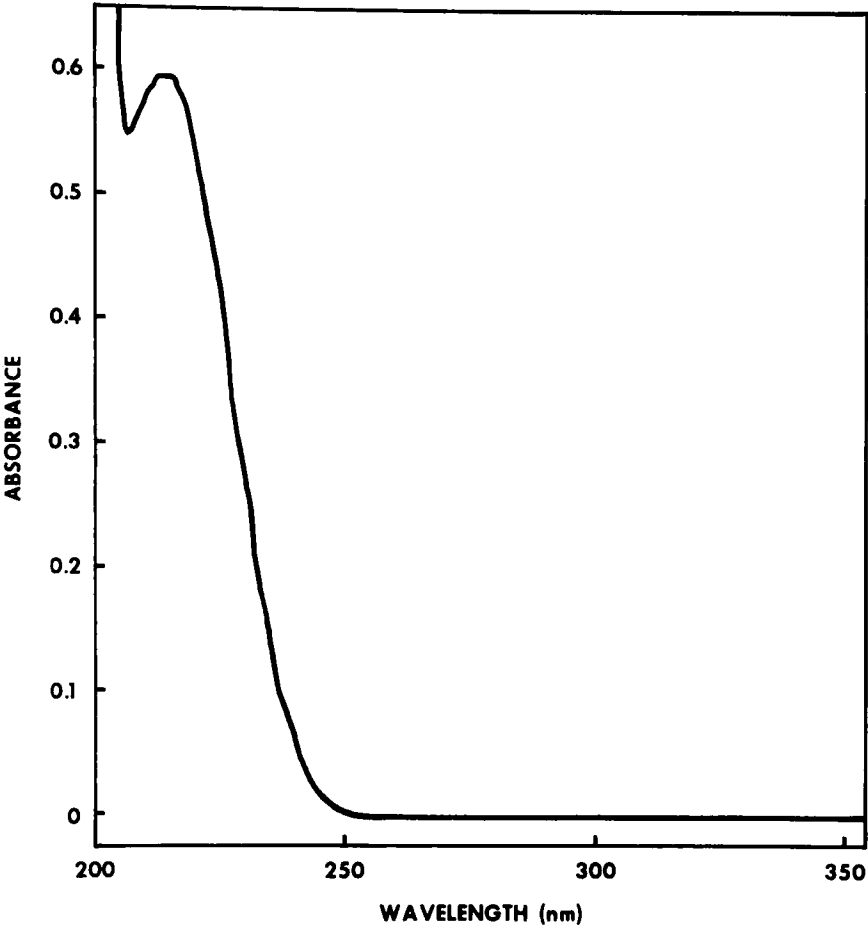


FIGURE 8 - RAMAN SPECTRUM OF VALPROIC ACID



**FIGURE 9 - ULTRAVIOLET SPECTRUM
OF VALPROIC ACID**



- a. $3000\text{--}2800\text{ cm}^{-1}$ Complex of strong bands due to the overlapping C-H stretching vibrations of the various methyl and methylene groups.
- b. 1660 cm^{-1} Weak band due to C=O stretching vibration of the carboxylic acid.
- c. 1450 cm^{-1} Due to the superimposing C-H bending vibrations of the various methyl and methylene groups.

2.5 Ultraviolet Spectrum (UV)

Sodium valproate in methanol solution has no UV maximum between 400 and 205 nm. When the UV spectrum of 0.1% solution of valproic acid in methanol solution was scanned from 400 to 205 nm, one maximum at 213 nm ($\epsilon = 86$) was observed (Figure 9). The spectrum was obtained with a Beckman Acta V Spectrophotometer.

2.6 Solubility

Approximate solubility data have been determined for sodium valproate at room temperature.

One gram of sodium valproate is soluble in 0.4 ml of water and also in 1.5 ml of ethanol. It is freely soluble in methanol (1 in 5). It is practically insoluble in common organic solvents such as ether, chloroform, benzene, n-heptane, etc.

The following solubility data have been determined for valproic acid at room temperature:

n-Heptane	Greater than 10%, v/v
Chloroform	Greater than 10%, v/v
Ethyl Acetate	Greater than 10%, v/v
Methanol	Greater than 10%, v/v
100% Ethanol	Greater than 10%, v/v
Acetone	Greater than 10%, v/v
Diethyl Ether	Greater than 10%, v/v
Benzene	Greater than 10%, v/v
1 N Aqueous NaOH	Greater than 10%, v/v
0.1 N Aqueous HCl	1.15 mg/ml
Water	1.27 mg/ml

2.7 Crystal Properties

The X-ray powder diffraction pattern of sodium valproate was determined by visual observation of a film obtained with a 143.2 mm Debye-Scherrer Powder Camera (Table IV). An Enraf-Nonius Diffractis 601 Generator; 38 KV and 18 MA with nikel filtered copper radiation; $\lambda = 1.5418$, was employed (4).

Table IV

X-Ray Powder Diffraction Pattern
d-Spacings and Intensities

<u>dA</u>	<u>I/I₁</u>	<u>dA</u>	<u>I/I₁</u>
16.0	60	2.80	5
13.4	100	2.66	2
7.7	50	2.61	1
6.7	3	2.57	1
5.8	5	2.46	1
5.25	20	2.42	1
4.9	5	2.37	2
4.77	2	2.23	2
4.42	5	2.20	2
4.21	30	2.06	2
4.09	35	2.01	1
3.95	5	1.97	1
3.85	1	1.93	2
3.64	15	1.90	1
3.40	20	1.86	1
3.20	1	1.81	5
3.13	3	1.75	1
3.02	4	1.69	1
2.87	5	1.66	1
2.84	5		

2.8 Dissociation Constants

Sodium valproate exhibits basic properties. Titration of an aqueous solution of sodium valproate with aqueous hydrochloric acid gave a pKa value of 4.8 (proton gained).

Titration of valproic acid with aqueous sodium hydroxide using acetone-water as the sample solvent and extrapolated to pure water gave a pKa value of 4.6 (proton lost).

2.9 Hygroscopic Behavior

Sodium valproate is hygroscopic. The rate of moisture absorption was tested in a humidity chamber using a Cahn Electro Balance and the results are shown in Table V (5).

Table V

Rate of Moisture Absorption
of Sodium Valproate

Relative Humidity	Time, min.						Overnight
	10	20	30	40	50	60	
12, 22 33, 43%	No gain						
53%	1.76	3.17	4.39	5.61	6.78	7.80	42.9%*

*Sample was completely liquified. Expressed as percent weight gain.

2.10 Sublimation

Sodium valproate did not sublime when it was stored at 105°C for 10 days.

2.11 Melting Range

Sodium valproate does not melt, decompose, or physically change form in the normal working range of the Thomas-Hoover Capillary Melting Point apparatus.

2.12 Boiling Point

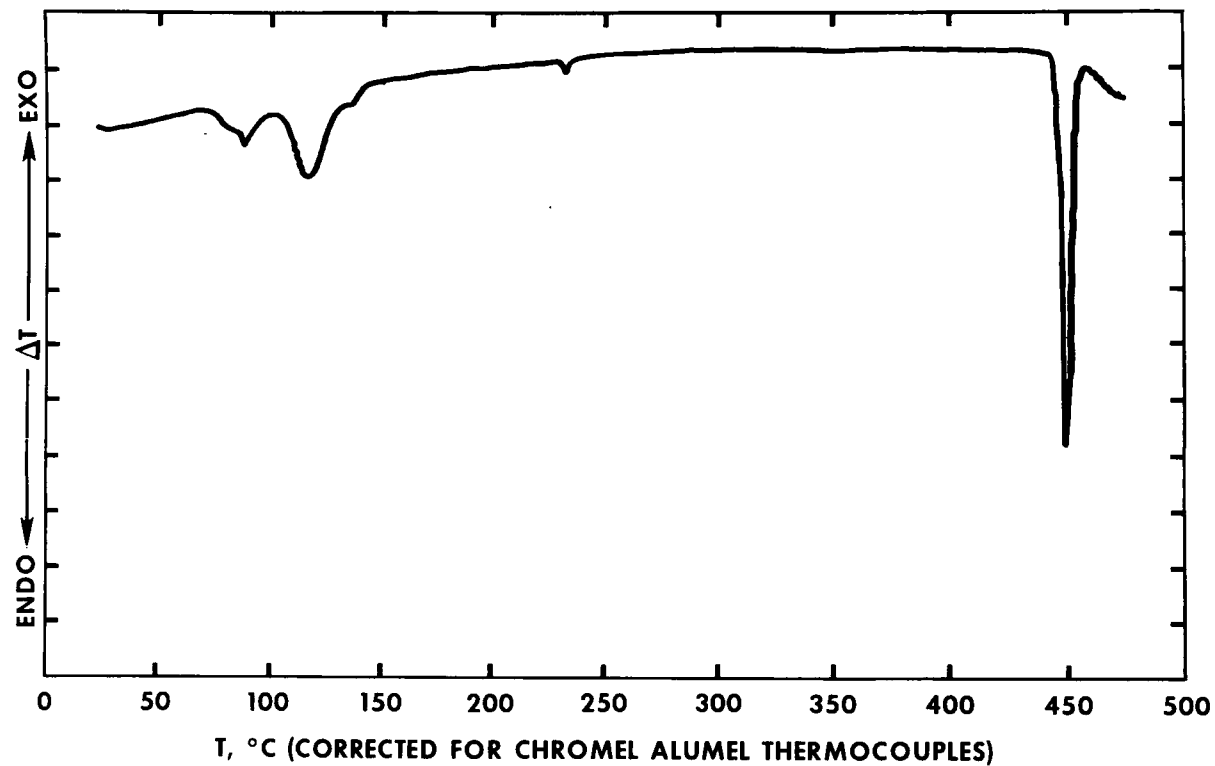
The following boiling points have been reported for valproic acid: bp₁₄ 120-121°C (6), bp₂₀ 128-130°C (6) and bp₇₆₀ 221-222°C (7).

2.13 Differential Thermal Analysis

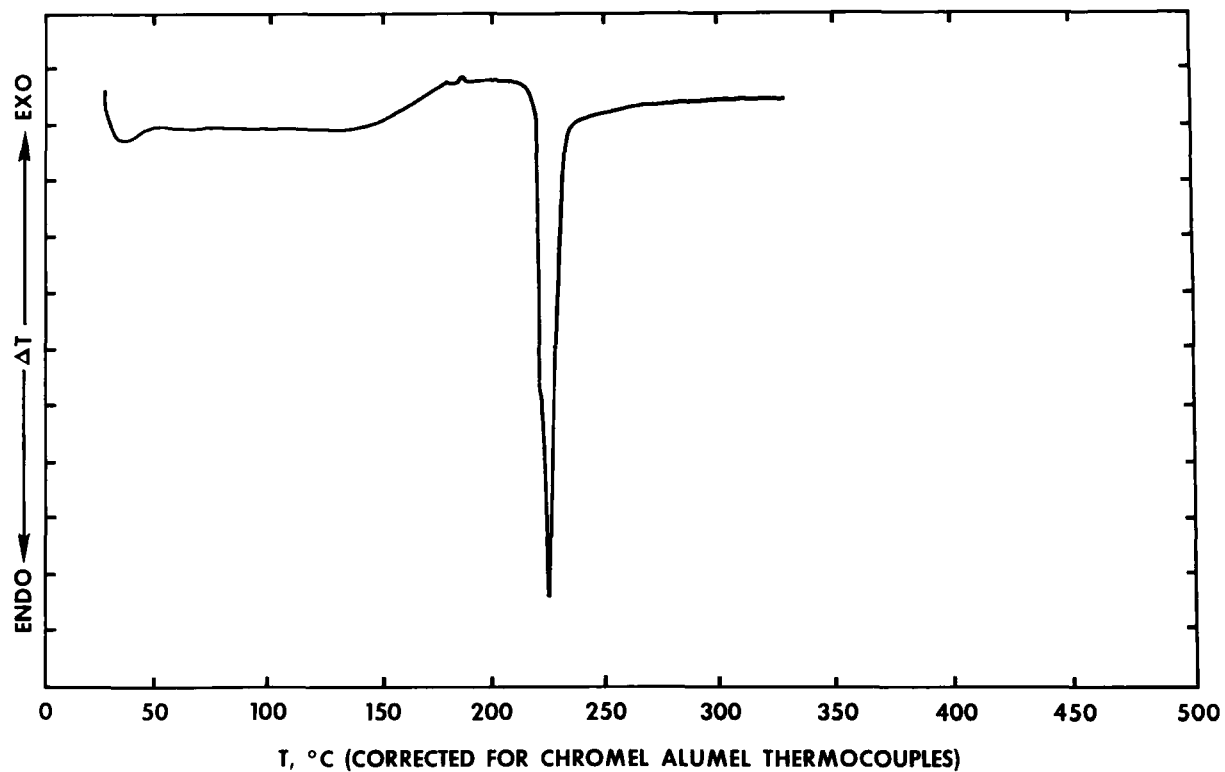
Differential thermal analysis of sodium valproate shows a large endotherm beginning at 100°C and ending at 118°C which is possible due to the loss of water. A sharp endothermic peak at 450°C is indicative of the melting point of sodium valproate.

Differential thermal analysis of valproic acid shows a sharp endothermic response at 225°C indicative of the boiling point of valproic acid.

**FIGURE 10 - DIFFERENTIAL THERMAL ANALYSIS CURVE
OF SODIUM VALPROATE**



**FIGURE 11 - DIFFERENTIAL THERMAL ANALYSIS CURVE
OF VALPROIC ACID**



2.14 Specific Gravity

The specific gravity of valproic acid was determined in a calibrated 25 ml pycnometer at 25°C. It has a value of 0.904 g/ml (8).

2.15 Refractive Index

Valproic acid has a refractive index of $n_D^{24.5}$ 1.425 (6).

3. Synthesis

The synthesis of valproic acid was first described in the literature by Oberreit (9) in 1896.

The sodium valproate is preferably formed from valproic acid by the interaction of sodium hydroxide in an aqueous solution. The synthetic pathways are shown in Figure 12.

4. Stability-Degradation

Sodium valproate was found to be extremely stable when refluxed in water, 1.0 N hydrochloric acid, or 1.0 N sodium hydroxide for 3 hours. Also, it was very stable when it was subjected to heat at 110°C for 10 days and to natural sunlight for 30 days in the dry state.

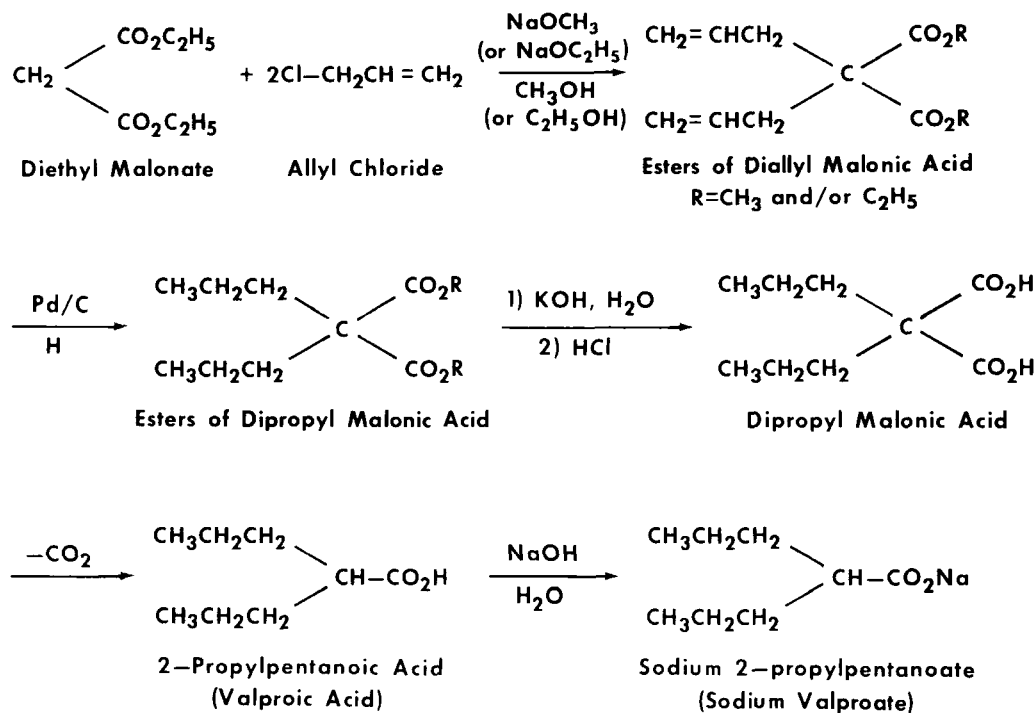
Valproic acid is a very stable compound. No degradation has been observed by the action of heat, light, and strong aqueous alkali, or acid.

5. Drug Metabolism and Pharmacokinetics

In 1971, Eymard et. al. (10) studied the distribution and the absorption of carbon-14 labeled sodium valproate in rats by oral administration.

The metabolites and metabolic pathway of a new anticonvulsant drug, sodium valproate, in rats were investigated using carbon-14 labeled sodium valproate. Most of the metabolites in urine and bile were a glucuronide conjugate of valproic acid. Free sodium valproate was as little as one-seventh of the total metabolites. In feces, only free sodium valproate was detected, and the possibility of enterohepatic circulation of sodium valproate was presumed. A part of dosed sodium valproate was excreted in expired air in the form of CO₂. This degradative reaction took place in liver mitochondria and required CoA and oxygen. It was stimulated by ATP

**FIGURE 12 - SYNTHETIC PATHWAYS OF VALPROIC ACID
AND SODIUM VALPROATE**



and EDTA, and inhibited by various enzyme reaction inhibitors such as malonate, Antimycin-A, chlorpromazine, p-chloromercuribenzoate (PCMB) and 2,4-dinitrophenol. Therefore, this degradation is not a one-step reaction, decarboxylation, but must be β -oxidation of a fatty acid. Thin-layer chromatography and gas-liquid chromatography were used for assay of metabolites (11).

The pharmacokinetics of distribution and elimination of sodium valproate in mice and dogs has been reported by Schobben and van der Kleijn (12).

The omega-oxidation of sodium valproate in rats has been reported by Kuhara, et. al. (13).

The absorption, excretion, and biotransformation of valproic acid were studied by Kukino and Matsumoto (14).

A preliminary pharmacokinetic profile of sodium valproate in monkey has been written by Levig, et. al. (15).

The pharmacokinetics of sodium valproate have been studied in 7 patients by Schobben, et. al. (16). The plasma concentrations were determined by gas-liquid chromatography during and following subchronic treatment. Elimination of the drug appeared to follow a monophasic exponential course; biological half lives were 8 to 15 hours. The drug appeared to have a relatively restricted distribution: calculated relative distribution volumes ranged from 0.15 to 0.40 l/kg. There were large interindividual differences in clearance rate. The therapeutic range was considered to be between 50 and 100 mg/l of plasma.

In 1976, Matsumoto, et. al. (17, 18) discovered several new metabolites of sodium valproate in rat urine, which support the hypothesis that the drug is also metabolized by a β -oxidation mechanism. One of the metabolites, 2-n-propyl 3-oxo-pentanoic acid, was recently found by Gompertz, et. al. (19) and Kochen, et. al. (20) to be a major constituent in urine of children who were receiving sodium valproate. The urinary 3-oxo derivative of valproate was reported to account for 20% of the administered dose.

6. Methods of Analysis

6.1 Identification

The presence of sodium cation may be identified by

a flame test. A positive test for sodium is produced in a non-luminous flame imparting a yellow color.

The presence of carboxylic anion may be identified by the following two tests:

- A. A 5% aqueous solution gives a violet precipitate with a 5% aqueous solution of cobalt nitrate.
- B. A 5% aqueous solution gives a violet precipitate with potassium iodobismuthite.

6.2 Elemental Analysis

A typical elemental analysis of a sample of sodium valproate is present in Table VI (21).

Table VI

Elemental Analysis of Sodium Valproate

<u>Element</u>	<u>% Theory</u>	<u>% Found</u>
C	57.82	57.82
H	9.10	9.38
O	19.25	-----*
Na	13.83	-----*

*The oxygen value cannot be determined due to presence of sodium.

A typical elemental analysis of a sample of valproic acid is present in Table VII (22).

Table VII

Elemental Analysis of Valproic Acid

<u>Element</u>	<u>% Theory</u>	<u>% Found</u>
C	66.63	66.39
H	11.18	11.32
O	22.19	22.43

6.3 Chromatographic Analysis

6.31 Thin-Layer Chromatography

A number of thin-layer chromatographic systems

on silica gel have been found to induce the migration of the compound. However, no system has been found which gives resolution equivalent to the gas-liquid chromatographic system detailed in section 6.32. Thin-layer chromatography is not the preferred method for determining the impurities. The following systems have been studied:

1. Chloroform:Methanol:Glacial Acetic Acid (17:2:1)
2. Ethyl Acetate:Formic Acid:Water (5:1:1)
3. Benzene:Methanol:Acetone:Ammonium Hydroxide (2:2:5:1)
4. Chloroform:Methanol:Ammonium Hydroxide (20:15:3)
5. n-Butanol Saturated with 10% Ammonium Hydroxide Solution (Aqueous)

6.32 Gas-Liquid Chromatography

The author has found the following GLC procedure to be suitable to determine the purity of sodium valproate and valproic acid. Preparatory to chromatography, the sodium valproate was acidified with a strong aqueous hydrochloric acid solution, and the valproic acid which is practically insoluble in water was separated. The separated free acid was then analyzed.

The following is the typical chromatographic condition for the GLC determination of valproic acid:

Column: 10% DEGS-PS on Supelcoport 80/100 mesh, Two 6 ft x 1/4 in o.d. stainless steel.

Detection: Thermal Conductivity Detector.

Temperature: Inj. Port 225°C
Column 160°C
Detector 300°C

Flow Rate: ~20 ml/min helium

Attenuation: 2 x 4

Slope Sensitivity: 0.03 or adjust for proper sensitivity

Sample Size: 2 μ l

Impurities as low as 0.01% could be detected using the above conditions.

6.4 Titrimetry

Sodium valproate exhibits basic properties. It can be titrated with 0.1 N hydrochloric acid.

In addition, sodium valproate can be potentiometrically titrated with standardized 0.1 N perchloric acid using a modified glass-calomel electrode system, in which 0.1 N lithium perchlorate in acetic acid has been substituted for potassium chloride, and employing glacial acetic acid as the sample solvent.

Valproic acid can be potentiometrically titrated with standardized 0.1 N tetra-n-butylammonium hydroxide in chlorobenzene using a modified glass-calomel electrode system, in which 1.0 M aqueous tetra-n-butylammonium chloride has been substituted for potassium chloride, and employing acetone as the sample solvent.

7. Determination of Valproic Acid and Its Metabolites in Biological Fluids

Many gas-liquid chromatographic methods for determination of valproic acid in biological fluids have been reported.

Early methods required derivatization of valproic acid (23, 24, 25, 26).

Although Meijer and Hessing-Brand in 1973 (27) developed a micro diffusion method without derivatization, it required special equipment.

Recently, most of the methods which have been used for the analysis of valproic acid in plasma, serum, cerebral spinal fluid, saliva, breast milk, and urine involve acidification of the biological sample, extraction into an organic solvent, and direct injection onto a gas-liquid chromatographic column (28, 29, 16, 30, 31, 32,

33, 34, 35, 36, 37, 38, 39).

Most of the methods employ an internal standard, and all are reported as accurate and reproducible. However, Schmidt, et. al. (40) recently reported great interlaboratory variation in the analysis of such biological samples.

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